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Key words:	Glycoalkaloids; *Solanum tuberosum*; α-Solanine; α-Chaconine, LC-TOF-MS; Natural Toxins; Quantification; Field Study; Potato; Dissipation; Degradation; Metabolites
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Preface

This Ph.D. thesis is submitted to University of Copenhagen, Faculty of Life Sciences in partial fulfillment of the Ph.D. degree. The work presented in this thesis is supported by a scholarship from the University of Copenhagen, Faculty of Life Sciences (LIFE) and organized in collaboration with the Geological Survey of Denmark and Greenland (GEUS). The work has been carried out at Department of Basic Sciences and Environment, LIFE and Department of Geochemistry, GEUS.

I would like to thank my three supervisors, Hans Christian B. Hansen and Bjarne W. Strobel, LIFE and Ole Stig Jacobsen, GEUS for qualified and positive support and supervision during the study. Special thanks to Ole Stig Jacobsen for valuable discussions of all kinds, guidance, and good company during the field work. Also, I would like to thank René K. Juhler, GEUS for the opportunity to use the LC-MS at GEUS when it was needed the most. I appreciate the work performed by the master student, Rasmus Broen Pedersen, and the fruitful discussions we have had during the work. Thanks to Torben Haugaard Jensen and Jacob Bælum for giving comments on this thesis.

Thanks to all the people working in the labs at both Department of Geochemistry and Department of Basic Sciences and Environment, who have always been willing to share their expertise and keep up the good spirit at all times. I have really enjoyed working in both departments. Acknowledgements are given to my office mates for many productive discussions about both serious and non-serious matters.

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Copenhagen, November 2008

Pia Haugaard Jensen, LC2384

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Pia H. Jensen, René K. Juhler, Nikoline J. Nielsen, Thomas H. Hansen, Bjarne W. Strobel, Ole S. Jacobsen, John Nielsen, Hans Christian B. Hansen (2008) *Potato glycoalkaloids in soil-optimising liquid chromatography–time-of-flight mass spectrometry for quantitative studies.* Journal of Chromatography A, 1182: 65–71.

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Manuscript IV

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Summary

Many plants and microorganisms produce toxins and there has been an increased interest in the fate of natural toxins in the terrestrial environment within the last decade. Several natural toxins have been detected in the soil or in surface, drainage, or soil water. The presence of natural toxins in the terrestrial environment is of concern, because they may have unintended effects on various organisms or because they may contaminate valuable drinking water resources. One of the most important crops in the world, the potato plant, produces the two toxic glycoalkaloids, α-chaconine and α-solanine. These compounds are present in all parts of the potato plant, and previous studies indicate that they may be relatively persistent in the terrestrial environment. Potato plants are often grown on sandy soils under heavy irrigation; both conditions increase the risk of leaching. Hence, the potato glycoalkaloids could possible be a risk in the terrestrial environment.

The aim of the Ph.D. work presented here was to investigate the fate of the potato glycoalkaloids, α-chaconine and α-solanine, in the soil and groundwater environment. As a part of the project, a sensitive and specific analysis method using liquid chromatography-electrospray ionization time-of-flight mass spectrometry (LC-ESI-MS-TOF) was developed in order to be able to detect the glycoalkaloids in environmental samples. The fate of the two glycoalkaloids in the environment was investigated in both laboratory and field studies. In the laboratory, degradation of the glycoalkaloids was studied in soil and groundwater; the latter enabled identification of formed degradation products. A conventional potato field was used in a field study, where the glycoalkaloid content was followed in plants, soil, and groundwater during one year. The present Ph.D. thesis consists of an introductory section and four manuscripts. One of the manuscripts is already published.

The **introduction section** consists of three major parts. The first part is a literature review of potato glycoalkaloids in general, with focus on the aspects relevant for their environmental fate. The second part comprises the analytical work. A review of the many methods, which are or have been used for determination of the glycoalkaloids, is given. Further, the analytical methods used in the present work are discussed in detail. The fate of the potato glycoalkaloids in the environment is discussed in the last part, and this section is primarily based on the results obtained in the present work. The fate studies are related to similar fate studies of other natural compounds.

Manuscript I describes the development of a sensitive LC-TOF-MS method for determination of the two potato glycoalkaloids and their common aglycone, solanidine, in environmental samples. The development included optimization of a new HPLC method and optimization of the response by the TOF-MS. Additionally, the fragmentation patterns of the compounds were presented. The performance of the method was evaluated with respect to linearity, precision, and detection limits. The obtained detection limits were in the range 2.2-4.7 µg/L. The work showed the LC-TOF-MS to be a powerful tool for quantitative studies of glycoalkaloids including identification of unknown metabolites.

Manuscript II is a lab degradation study of α-chaconine and α-solanine in groundwater sampled from the field location at Fladerne Bæk, Denmark. The degradation of the glycoalkaloids and the

formation of metabolites were followed by LC-MS. The degradation was shown to be primarily microbial and proceeded as a cleavage of the three carbohydrate units. The metabolites, β_1-solanine, γ-solanine, and solanidine were formed from α-solanine, while β-chaconine, γ-chaconine, and solanidine were detected from α-chaconine. This is the first report of the formation of β_1-solanine by microbial degradation. The metabolite, solanidine, was also degraded, but no further metabolites could be detected. Thus, this study shows that indigenous groundwater microorganisms are capable of degrading the glycoalkaloids.

Manuscript III is a comprehensive field study. The glycoalkaloid content in potato plants, soil, and groundwater from a potato field was followed during a growth season and the following winter. The field location was a sandy soil from Fladerne Bæk, Denmark used for potato growing. In the plants, the maximum glycoalkaloid concentration of 22 g/kg dry weight was found in June. The total plant amount of glycoalkaloids was at maximum in July (25 kg/ha), after which it decreased during plant senescence to below 0.63 kg/ha in October. In the upper soil, glycoalkaloids were found in concentrations of up to 2.8 mg/kg dry weight. The highest soil load was estimated to be 0.6 kg/ha in September. Glycoalkaloids were still present in the soil in March, despite no further transfer from the plants during winter. Hence, the dissipation in the soil was slow during winter. The amount of glycoalkaloids found in the soil accounted for only a minor fraction of the amount present in the plants. The investigation also showed degradation of the glycoalkaloids in the potato plant during decay. Thus, the major dissipation route for the glycoalkaloids was proposed to be degradation within the plant material. No traces of glycoalkaloids were detected in the groundwater sampled from 2-4 m below the potato field during the growth season. From these results, the leaching potential of the glycoalkaloids is evaluated to be low.

In **Manuscript IV**, the degradation of the potato glycoalkaloid, α-solanine, was followed in three agricultural soils, including soil from Fladerne Bæk. Similar degradation pattern was found in all soils, where a fast initial degradation was followed by a slower phase. The pattern was well described by a sum of two first-order expressions. Half-lives ranging from 1.8-4.1 days were found for the three topsoils at 15 ºC, but residuals were still detected by the end of the experiment after 42 days. For the Fladerne Bæk soil, the degradation was additionally followed at 5 ºC in both top- and subsoil and here half-lives of similar lengths ranging from 4.7-8.7 days were found. Overall, fast degradation was found in both top- and subsoil even at low temperatures, and from these results, the risk of glycoalkaloid leaching to the groundwater appears to be limited.

Resumé (Danish Summary)

Mange planter og mikroorganismer producerer toksiner, og i løbet af de seneste år er der opstået en øget interesse for naturlige toksiners skæbne i det terrestriske miljø. Således er der rapporteret om flere fund af naturlige toksiner i jord samt i overflade-, dræn- eller jordvand. De naturlige toksiners tilstedeværelse i det terrestriske miljø kan give anledning til bekymring, enten fordi forbindelserne kan forårsage uønskede effekter på forskellige organismer, eller fordi de kan forurene værdifulde drikkevandsressourcer. En af de vigtigste afgrøder i verden, kartoffelplanten, producerer to toksiske glykoalkaloider, α-chaconin og α-solanin. Disse to forbindelser findes i alle dele af kartoffelplanten, og de hidtidige studier af glykoalkaloiderne indikerer, at de kan være relativt persistente i det terrestriske miljø. Dyrkning af kartofler foregår ofte på sandede jorde med stor markvanding. Dette er begge forhold, som øger risikoen for udvaskning. De samlede omstændigheder medfører, at kartoffelglykoalkaloiderne kan udgøre en mulig risiko i det terrestriske miljø.

Formålet med nærværende projekt var at undersøge skæbnen for de to kartoffelglykoalkaloider, α-solanin og α-chaconin, i jord- og grundvandsmiljøet. En del af projektet bestod i at udvikle en følsom og specifik analysemetode til bestemmelse af glykoalkaloiderne i prøver fra miljøet. Metoden blev udviklet ved brug af væske kromatografi – elektrospray ionisering – time-of-flight – masse spektrometri (LC-ESI-TOF-MS). Studier i laboratoriet og i felten har begge indgået i undersøgelsen af glykoalkaloidernes skæbne i miljøet. I laboratoriet blev nedbrydningen af forbindelserne i jord og grundvand undersøgt, og endvidere blev dannelsen af metabolitter fulgt i grundvand. I feltstudiet blev indholdet af glykoalkaloider i planter, jord og grundvand fra en konventionel kartoffelmark fulgt gennem et år. Denne Ph.D. afhandling består af en introduktion samt af fire artikelmanuskripter, heraf er én artikel allerede publiceret.

Introduktionen består af tre hoveddele. Første del er en gennemgang af den tilgængelige litteratur vedrørende kartoffelglykoalkaloider. I gennemgangen lægges der vægt på de aspekter, der er relevante i forhold til stoffernes skæbne i miljøet. I anden del behandles de analytiske metoder, og der er en gennemgang af den lange række af analysemetoder, som enten bruges eller tidligere har været brugt til bestemmelse af glykoalkaloiderne. De benyttede analysemetoder er desuden behandlet i detaljer. I sidste del diskuteres kartoffelglykoalkaloidernes skæbne i miljøet, og der er her fokuseret på dette projekts undersøgelser. Disse undersøgelser relateres til tilsvarende studier af andre naturstoffer.

Manuskript I beskriver udviklingen af en følsom LC-TOF-MS metode til bestemmelse af de to glykoalkaloider og deres fælles aglykon, solanidin, i prøver fra miljøet. Metodeudviklingen omfattede optimering af en ny HPLC metode samt optimering af det opnåede respons på TOF-MS. Fragmenteringsmønsteret for de tre forbindelser blev også vist. Metodens kvalitet blev evalueret med hensyn til linearitet, præcision og detektionsgrænse. De opnåede detektionsgrænser lå i området 2.2-4.7 µg/L. HPLC-TOF-MS har i dette arbejde vist sig at være et stærkt instrument i forhold til at udføre kvantitative bestemmelser af glykoalkaloider samt til identifikation af tilhørende ukendte metabolitter.

Manuskript II er et nedbrydningsstudie af α-chaconin og α-solanin i grundvand, udtaget fra feltlokaliteten, Fladerne Bæk, Danmark. Nedbrydningen af glykoalkaloiderne og dannelsen af metabolitter blev fulgt på LC-MS. Det blev vist, at nedbrydningen primært foregik mikrobielt, hvor nedbrydningen skete via en kløvning af de tre kulhydratenheder. Metabolitterne, β_1-solanin, γ-solanin og solanidin, blev dannet fra α-solanin, mens der foregik en dannelse af β-chaconin, γ-chaconin og solanidin fra α-chaconin. Det er første gang, at en mikrobiel dannelse af β_1-solanin er blevet rapporteret. Den dannede metabolit, solanidine, blev også nedbrudt, men det var ikke muligt at bestemme efterfølgende metabolitter. Dette studie viser, at grundvandets iboende mikroorganismer er i stand til at nedbryde glykoalkaloiderne.

Manuskript III er et omfattende feltstudie. Indholdet af glykoalkaloider i kartoffelplanter, jord og grundvand fra en kartoffelmark blev fulgt gennem en vækstsæson og den efterfølgende vinter. Feltlokaliteten var en sandet kartoffelmark fra Fladerne Bæk, Danmark. Den højeste koncentration af glykoalkaloider på 22 g/kg tørstof blev fundet i planterne i juni. Derimod var den totale mængde af glykoalkaloider i planterne på det højeste niveau i juli (25 kg/ha), hvorefter den faldt, da planterne visnede og endte på under 0.63 kg/ha i oktober. I det øverste jordlag blev der fundet koncentrationer af glykoalkaloider på op til 2.8 mg/kg tørstof. Det højeste niveau i jorden blev estimeret til 0.6 kg/ha i september. Glykoalkaloiderne var stadig til stede i jorden i marts måned, selv om der ikke var blevet tilført yderligere mængder fra planterne henover vinteren. Forsvindingen i jorden om vinteren foregik således langsomt. Samlet set, udgjorde den fundne mængde glykoalkaloider i jorden kun en lille del af, hvad der var målt i planterne. Desuden viste undersøgelsen, at der foregik en nedbrydning af glykoalkaloiderne i kartoffelplanten, når den visnede. Derfor menes det, at den primære forsvinding af glykoalkaloiderne fra miljøet foregik via nedbrydning i selve planten. Der blev i løbet af vækstsæsonen ikke fundet spor af glykoalkaloider i grundvandet, som var udtaget fra en dybde på 2-4 m under kartoffelmarken. Ud fra de opnåede resultater vurderes det, at glykoalkaloidernes udvaskningspotentiale er lille.

I **Manuskript IV** følges nedbrydningen af glykoalkaloidet, α-solanin, i tre landbrugsjorde, inklusiv jorden fra Fladerne Bæk. Nedbrydningen fulgte et ensartet mønster i alle jordene, hvor der indledningsvis foregik en hurtig nedbrydning. Denne blev fulgt af en periode med langsommere nedbrydning. Nedbrydningskinetikken blev beskrevet med en sum af to første ordens udtryk. Der blev fundet halveringstider i størrelsesordenen 1.8-4.1 dage for de tre overjorde ved 15 ºC, men samtidig kunne der stadig måles rester af forbindelserne ved slutningen af forsøget efter 42 dage. I både over- og underjorden fra Fladerne Bæk blev nedbrydningen desuden fulgt ved 5 ºC. Her var halveringstiderne i størrelsesordenen 4.7-8.7 dage. Samlet set blev der observeret en hurtig nedbrydning i både over- og underjord – selv ved lav temperatur, og resultaterne indikerer, at der er en begrænset risiko for udvaskning af glykoalkaloiderne til grundvandet.

Abbreviations

a	constant
AOAC	Association of analytical communities
ASE	accelerated solvent extraction
C_0	concentration of a compound at t = 0
CE	capillary electrophoresis
C_{soil}	concentration of a compound in the soil
C_t	concentration of a compound at t
C_{water}	concentration of a compound in the soil solution
DT_{50}	dissipation time for 50% of the applied compound
DT_{90}	dissipation time for 90% of the applied compound
DW	dry weight
ESI	electrospray ionization
f_{oc}	fraction of organic carbon in soil
FW	fresh weight
GC	gas chromatography
HPLC	high performance liquid chromatography
k	rate constant
K_d	linear distribution coefficient
K_f	Freundlich distribution coefficient
K_{oc}	organic carbon normalized distribution coefficient
K_{ow}	octanol-water distribution coefficient
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption/ionization
MRM	multiple reaction monitoring
MS	mass spectrometer / mass spectrometry
n	constant
pK_a	negative logarithm to the acid dissociation constant
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	selected reaction monitoring
t	time
TLC	thin layer chromatography
TOF	time of flight
UV	ultraviolet light
V_p	vapor pressure

1 Introduction

A vast number of toxins are produced by plants and microorganisms and alone in the human diet 5,000-10,000 natural toxins are estimated to be present (Ames et al., 1990). The group of natural toxins are diverse in terms of structure, toxicity, and properties (Teuschler and Lindequist, 1988). Many of the toxins are well-known and well-studied; either because the compounds have found use as e.g. natural pesticides or medicine or because of their presence in important food or feed. One example is *Artemisia annua*, which is now cropped in large scale, because the plant produces a compound with antimalarial effect, artemisinin (Ferreira et al., 2005). Strychnine produced by the strychnine tree (*Strychnos nux vomica*) is an example of a compound now used as a rodenticide (Starr et al., 1996). Further, the bracken fern (*Pteridium aquilinium*) is an example of a plant on which animals may be browsing; this plant contains the toxic compound, ptaquiloside (Rasmussen et al., 2005).

The spreading of chemicals in general and of toxic substances in particular to the terrestrial environment is of great concern, because it may have unintended effects on soil organisms or plant growth or because it through leaching may reach surface or ground water, where it can contaminate valuable drinking water resources or affect aquatic organisms. Hence, knowledge of the fate of these compounds in the environment is needed. Comprehensive fate studies for various groups of compounds as e.g. the pesticides have been performed; e.g. Sarmah et al. (2004) has given an overview of the work on pesticides carried out just under New Zealand conditions. For pesticides in drinking water, a general limit of 0.1 µg/L (for each individual active compound) is set by EU (European Community, 1998) and programs such as the national Danish ground water monitoring program are well-established in order to monitor a range of xenobiotic compounds. Pesticide findings above the limit have led to closure of Danish drinking water supplies. However, the group of natural toxins is sparely studied in terms of environmental fate. In spite of the well-known toxicity of the compounds, only little is known about their fate and possible effect in the terrestrial environment. Recent studies have revealed that some of the natural toxins produced by plants or microorganisms can also be detected in soil or in surface, drainage, or soil water in the surroundings. One example is the compound produced by the bracken fern, ptaquiloside, which has been found in deeper soil layers, soil water, and surface wells (Rasmussen et al., 2005; Engel et al., 2007). Another example is two mycotoxins, deoxynivalenol and zearalenone, which were detected in drainage water from a field inoculated with *Fusarium graminearum* infected winter wheat (Bucheli et al., 2008). These compounds were additionally detected in river water in very low concentrations. Hence, natural toxins may not only be released to the terrestrial environment, but they may also persist long enough for transport through the soil matrix to the surrounding water bodies. Depending on the toxicity and the concentration of the compound, this may possibly result in an effect on terrestrial or aquatic organisms or on humans.

The potato plant is one of the most important crops in the world. More than 321 million tones were produced worldwide in 2007 using 19 million hectare; in Denmark alone 1.5 million tones were produced on 42,000 hectare (FAO, 2008). Potatoes are often grown on sandy soils with low water

holding capacity. These soils are in general vulnerable to leaching, because they contain little sorption material. The potato fields are heavily irrigated, which will result in large percolation and lead to an increased risk of leaching. The potato plant produces two glycoalkaloids, α-chaconine and α-solanine, which are known to be toxic to human as well as many other organisms including fungi, snails, and insects (McKee, 1959; Morris and Lee, 1984; Fewell and Roddick, 1993; Roddick, 1996; Smith et al., 2001). The compounds are present in all parts of the potato plant, where the highest concentrations are found in the above ground plant material (Friedman and McDonald, 1997; Kolbe and Stephan-Beckmann, 1997a; Kolbe and Stephan-Beckmann, 1997b). The glycoalkaloids are partly metabolized by some pathogenic fungi (e.g. Weltring et al., 1997), but only one report of conversion by non-pathogens has been published (Oda et al., 2002). Furthermore, the glycoalkaloids do not hydrolyze easily in aqueous solutions (Friedman and McDonald, 1995b) and they are little affected by heating processes (Bushway and Ponnampalam, 1981). Overall, this indicates that the glycoalkaloids could be relatively persistent in the environment.

The high biomass of the potato plant and the high amount of glycoalkaloids in the plant result in a high potential glycoalkaloid load to the soil environment from a potato field. The circumstances, under which the potato plants are grown, constitute a general high risk of leaching. The possible persistence of the compounds in the environment may increase the risk of leaching, because of the prolonged lifetime in the soil. Overall, in addition to the worldwide importance of this crop, this is the motivation for the investigation of the fate of the glycoalkaloid in the environment.

The aim of the Ph.D. work presented here was to investigate the fate of the potato glycoalkaloids in the soil and groundwater environment. To perform the investigation, a new sensitive and structure-specific analysis method for determination of the glycoalkaloids was developed. The investigation comprised of both lab and field studies where the fate of the potato glycoalkaloids in plant, soil, and groundwater was followed. The major part of the work, including the field study, was performed using soil and groundwater from a typical sandy field used for potato growing located at Fladerne Bæk, Karup, Jutland, Denmark. The present thesis consists partly of a synopsis and partly of four enclosed manuscripts. In the synopsis, the theoretical background for the work is given and the literature available is discussed. The results presented in the four manuscripts and from unpublished work are included and placed into context. Other glycoalkaloids are drawn into the discussions as support for the limited data available for the potato glycoalkaloids, when appropriate. The obtained results relating to fate are compared with similar studies of other natural toxins.

2 Potato Glycoalkaloids

2.1 Glycoalkaloids

The potato glycoalkaloids belong to the group of alkaloids, which is defined as a group of nitrogen-containing organic compounds of natural origin with a greater or lesser degree of basic character (Hesse, 2000). The alkaloids are an enormous and diverse group of secondary metabolites; in fact, more than 12,000 alkaloids have been described (Wink, 1998). Examples of well-known alkaloids are strychnine, cocaine, caffeine, nicotine, and α-solanine. The huge group of alkaloids is divided into a number of groups with more similar structures, and the potato glycoalkaloids belong to the group of steroidal glycoalkaloids, which are all glycosidic derivatives of nitrogen-containing steroids. The steroidal glycoalkaloids are produced in more than 350 plant species, mainly of the families *Solanaceae* and *Liliaceae* (Roddick, 1996). They consist of a C27 cholestane skeleton to which a carbohydrate moiety of one to five monosaccharides is attached (van Gelder, 1991; Roddick, 1996). More than 75 different naturally occurring aglycone structures are known (Roddick, 1996), and it will be beyond the scope of this work to go into detail with all these different compounds.

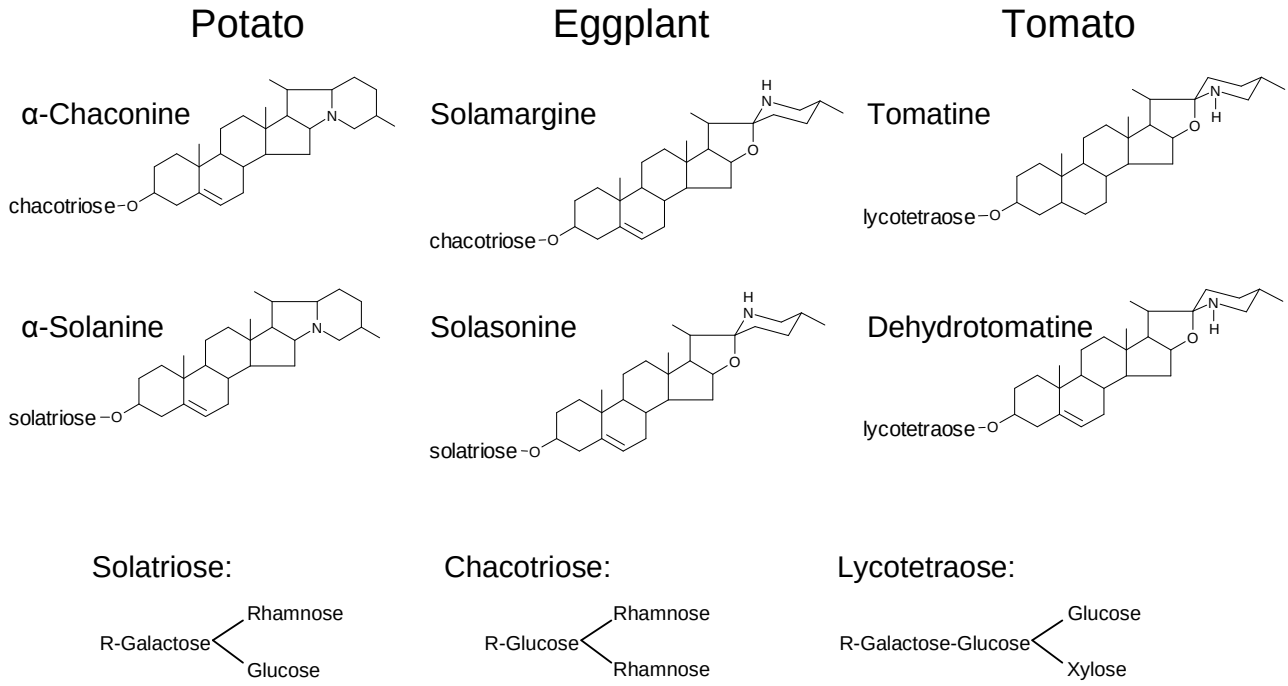

Figure 2.1 Structures of potato, tomato, and eggplant glycoalkaloids.

Among the well-known species of the family *Solanaceae* are the potato plant (*Solanum tuberosum*), the eggplant (*Solanum melongena*), and the tomato plant (*Lycopersicon esculentum*), which all produce glycoalkaloids. Each of the three species produce mainly two very similar glycoalkaloids (Friedman, 2002; Friedman, 2006) (**Figure 2.1**). The potato plant produces the glycoalkaloids, α-chaconine and α-solanine, which share a common aglycone, solanidine, to which a trisaccharide moiety; either chacotriose (α-chaconine) or solatriose (α-solanine), is attached. Similar units are

attached to the aglycone, solasodine produced by the eggplant, hereby producing the glycoalkaloids, solamargine and solasonine. The tomato plant produces the compounds, α-tomatine and dehydrotomatine, which only differ by the presence or absence of a double bond in the ring structure. In domestic potato plants, α-solanine and α-chaconine are usually the two dominating glycoalkaloids (Friedman, 2006), but several other glycoalkaloids may be present as well. Especially in the wild species, a higher diversity of glycoalkaloids can be found. A resent study of potato glycoalkaloids with a solanidine-like aglycone in four wild potato species and three cultivars revealed more than 50 different glycoalkaloids, of which the majority was only present in the wild species (Shakya and Navarre, 2008). This thesis focuses mainly on the two potato glycoalkaloids, α-chaconine and α-solanine, and in the following, the term *glycoalkaloids* is used synonymous with these two compounds.

2.2 Physical and Chemical Properties

Selected physical and chemical properties of α-chaconine, α-solanine and their aglycone, solanidine, are shown in **Table 2.1**. Because the experimental data for the glycoalkaloids are limited, some properties have been computer estimated by use of EPIwin software (USEPA, 2007). All of the three compounds have low water solubility and contain a tertiary amine, which is protonated at lower pH. The estimated log K_{ow} values differ widely between α-solanine and α-chaconine, and the difference origins from the weight the different carbohydrate moieties are given in the estimation. This also influences the difference between the two estimated water solubilities, because the estimated log K_{ow} values are used for this estimation. It was, during the experimental work, also observed that α-chaconine is less water soluble than α-solanine, the size of the difference between the two estimated log K_{ow} values may however be uncertain.

Table 2.1 Physical and chemical properties of the potato glycoalkaloids, α-chaconine and α-solanine, and their common aglycone, solanidine.

	α-chaconine	**α-solanine**	**solanidine**
CAS no.	20562-03-2	20562-02-1	80-78-4
Molecular formula	$C_{45}H_{73}NO_{14}$	$C_{45}H_{73}NO_{15}$	$C_{27}H_{43}NO$
Molar mass (g/mol)	852.1	868.1	397.6
Water solubility (mg/L)	0.0021 (est)[a]	1.38 (est)[b]	0.00131 (est)[b]
Log K_{ow}	4.9 (est)[a]	2.0 (est)[b]	7.3 (est)[b]
Log K_{oc}	4.1 (est)[a]	4.3 (est)[a]	6.1 (est)[a]
V_p (mm Hg)	6.78×10^{-33} (est)[a]	1.67×10^{-34} (est)[b]	2.89×10^{-11} (est)[b]
pK_a		6.7[c]	8.6[d]

[a] Calculated with EPIwin v3.20 (USEPA, 2007)
[b] SRC, 2008
[c] Budavari, 1996
[d] Bloom and Briggs, 1952

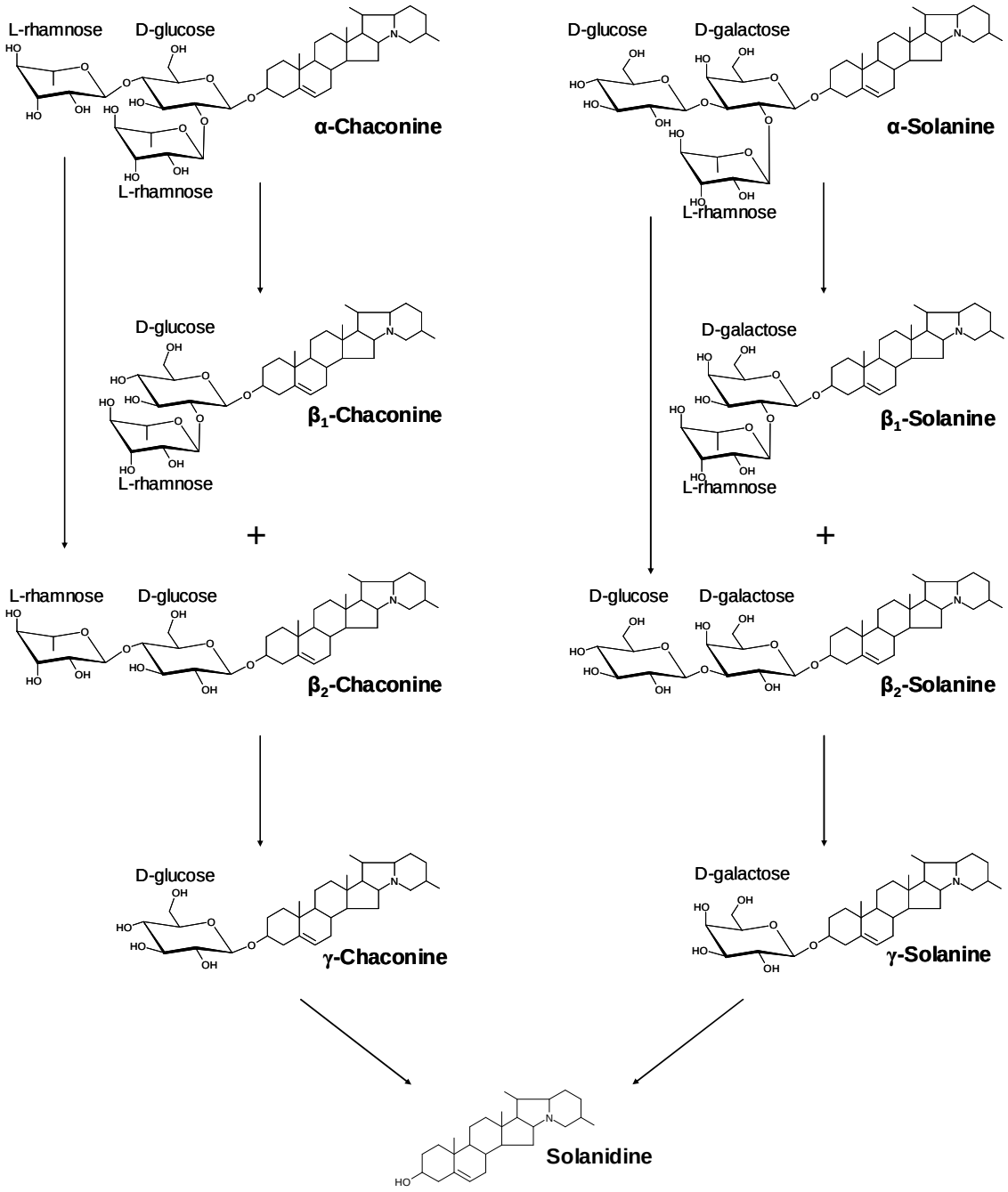

Figure 2.2 Possible hydrolysis and degradation products of the potato glycoalkaloids.

The glycoalkaloids are rather stable compounds; e.g. no or little loss is observed when the potato tubers are exposed to various cooking procedures such as baking, microwaving, boiling, or frying (Bushway and Ponnampalam, 1981). The glycoalkaloids may undergo acidic or enzymatic hydrolysis, whereby the side chain of carbohydrates is partly or fully removed (**Figure 2.2**). Hereby, compounds containing two (β-compounds), one (γ-compounds) or no carbohydrates (solanidine) are produced. β_1- and β_2-chaconine have the same molar mass and can thus not be distinguished by mass, why, if no differentiation has been done, both compounds are sometimes just mentioned β-chaconine. The enzymatic processes are discussed in detail in Chapter 5. Acidic hydrolysis is favored by an alcoholic solvent, whereas an increased proportion of water in the

solution reduces the rate (Friedman and McDonald, 1995b). Higher acid concentration and higher temperature increase the hydrolysis rate as well (Friedman et al., 1993). α-Chaconine is faster hydrolyzed and undergoes acid catalyzed hydrolysis under milder conditions than α-solanine (Friedman et al., 1993; Friedman and McDonald, 1995b). E.g. 10% α-chaconine and 2% α-solanine were hydrolyzed at 38 ºC in a methanolic 0.2M HCl solution after 90 minutes, while the amount increased to 79% and 46%, respectively at 65 ºC (Friedman et al., 1993). In a study, where the conditions were optimized in order to obtain a maximal production of hydrolysis products, four hydrolysis products (β_1-chaconine, β_2-chaconine, γ-chaconine and solanidine) were obtained from α-chaconine (Friedman and Levin, 1992; Friedman et al., 1993). In contrast, only three products (β_2-solanine, γ-solanine and solanidine) were found from α-solanine (Filadelfi and Zitnak, 1983; Friedman et al., 1993). Small amounts of other unknown hydrolysis products were observed as well, but β_1-solanine was not detected (Friedman et al., 1993).

The aglycone, solanidine, is an important precursor for hormone synthesis, and therefore Nikolić and Stanković (2003) optimized a method for extraction, hydrolysis and isolation of solanidine in one step. In this system, hydrolysis was performed in 10% (w/v) hydrochloric acid in 50% methanol, after which solanidine was transferred to a phase of chloroform. Under these conditions, more than 98% of the glycoalkaloids were transformed to solanidine after 90 minutes of extraction.

In aqueous solutions, the hydrolysis is slower. Under conditions present in the digestive tracts of animals (1 M HCl, 37 ºC), few percentages of the potato glycoalkaloids were hydrolyzed within 3 h (Friedman et al., 1993), and similar results were obtained for the tomato glycoalkaloid, α-tomatine (Friedman et al., 1998b). In **Manuscript IV**, hydrolysis was studied at pH 3-6 (20 ºC). Here no hydrolysis was observed within 29 days.

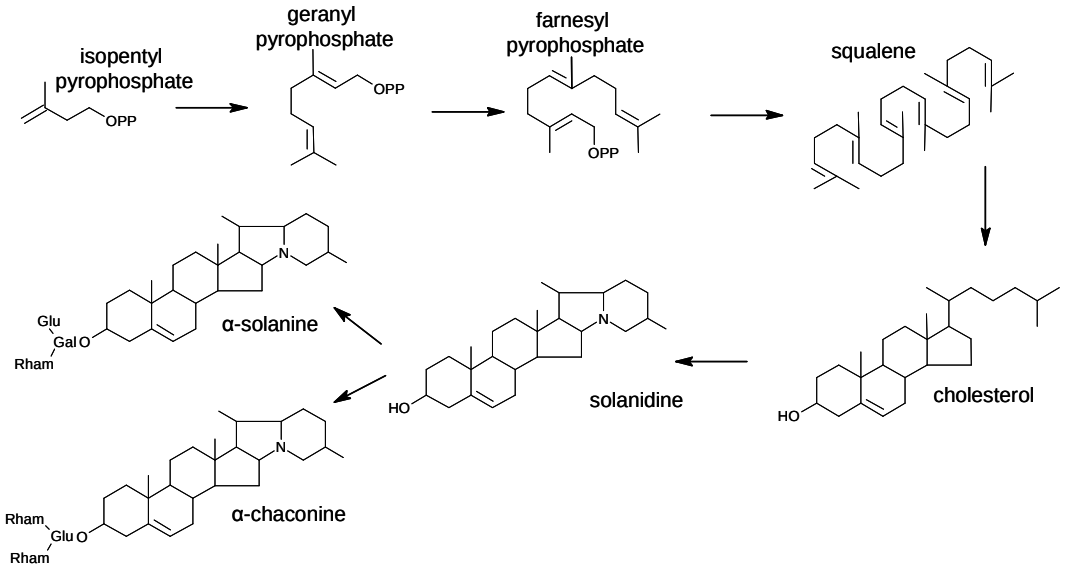

Figure 2.3 The biosynthetic pathway to the two potato glycoalkaloids, α-chaconine and α-solanine.

2.3 Biosynthesis

The glycoalkaloids are synthesized in all parts of the potato plant (Friedman, 2006), and the biosynthesis may continue in the tubers after harvest (Friedman and McDonald, 1997). The biosynthesis is described in detail in Heftmann (1983) and Bergenstråhle (1995); a short summary is given here. The biosynthesis proceeds via the mevalonic pathway, which in general is responsible for the steroid production (Friedman and McDonald, 1997) (**Figure 2.3**). Intermediates during the biosynthesis are acetate (C2), mevalonate (C6), isopetenyl pyrophosphate (C5), geranyl pyrophosphate (C10), farnesyl pyrophosphate (C15), squalene (C30), and cholesterol (C27). From cholesterol, solanidine is formed, after which after the carbohydrate moieties are attached.

Table 2.2 Glycoalkaloid content of different parts of the potato plant.

Plant part	Total glycoalkaloid content (mg/kg FW)[a]	Mass ratio of α-chaconine to α-solanine	Reference
Stems	320-450[b]	1.4-1.6	(Friedman and Dao, 1992)
Leaves and stem	54-4,090[c]	0.2-3.0[c]	This project, partly in **Manuscript III**
Leaves	1,450[b]	1.4	(Friedman and Dao, 1992)
	320-1,270[b]	2.0-2.8	(Dao and Friedman, 1996)
	230-590	1.2-1.4	(Kozukue et al., 1987)
Flowers	3,000-5,000	0.7	(Kozukue et al., 1987)
Berries	380	1.4	(Friedman and Dao, 1992)
	180-1,350	ND[d]	(Coxon, 1981)
	340	2.1	**Manuscript III**
Roots	860[b]	1.1	(Friedman and Dao, 1992)
Sprouts	2,750-10,000[b]	1.1-1.2	(Friedman and Dao, 1992)
Tubers	150[b], 18-57	1.6-2.0	(Friedman and Dao, 1992)
	7-190	1.4-2.2	(Friedman et al., 2003b)
Peel	850[b]	2.7	(Friedman and Dao, 1992)
	12-540	1.4-2.4	(Friedman et al., 2003b)
Flesh	120[b]	2.0	(Friedman and Dao, 1992)
	1-150	1.2-2.6	(Friedman et al., 2003b)
Tubers left in the field	390	1.4	**Manuscript III**

[a] Fresh weight

[b] High glycoalkaloids-containing plant

[c] Based on individual plants, determined in plants during the growth season. A general decline in concentration and ratio was observed during the growth season.

[d] ND not determined

2.4 Occurrence

Glycoalkaloids are found in all parts of the potato plant and there is little or no transport of the compounds between the plant parts (Friedman and McDonald, 1997; Friedman, 2006). The highest concentrations are detected in the very actively growing young tissues as flowers, sprouts and young leaves (Friedman and McDonald, 1997); an overview of concentrations found in different plant parts is presented in **Table 2.2**. Relatively low concentrations are usually found in the tubers; the highest concentration is present in the outermost few millimeters, while the flesh contains lower concentrations (Kozukue et al., 1987). Small tubers are often found to contain higher concentrations (Papathanasiou et al., 1999); this may be related to the higher peel to flesh ratio in the small tubers. The proportion between α-solanine and α-chaconine may vary between tissue types and varieties (**Table 2.2)**; ratios of 0.2-3.0 are observed. In most cases, α-chaconine is present in a slightly higher amount.

Similar distributions of glycoalkaloids between tissue types are found in other glycoalkaloid containing plant species. In the two other *Solanum* species, *S. nigrum* and *S. incanum*, the highest content of solasodine was detected in the leaves (up to 4 g/kg dry weight), with the highest concentrations found in small leaves, while lower levels were found in fruits, roots, and stem (Eltayeb et al., 1997). In tomato plants, α-tomatine and dehydrotomatine were also found in all of the different plant tissues. The highest concentrations were found in flowers and in some cases in the green fruits (up to 18 g/kg FW), while especially in red mature fruits much lower concentrations were detected, due to degradation of the glycoalkaloids during ripening (Friedman and Levin, 1995; Kozukue et al., 2004).

The glycoalkaloid concentration in the potato tissues changes during growth. A whole range of experimental data were collected and analyzed by Kolbe and Stephan-Beckmann (1997a; 1997b) in order to develop universal statements concerning the development of the potato plant. They reported a decrease in glycoalkaloid concentration in leaves and stem of about 20% after four months of growth. A more distinct decrease for leaves and stem combined was shown in the present study (**Manuscript III**); where the concentrations in October comprised only approximately 1% of the concentration found in June (**Figure 2.4**).

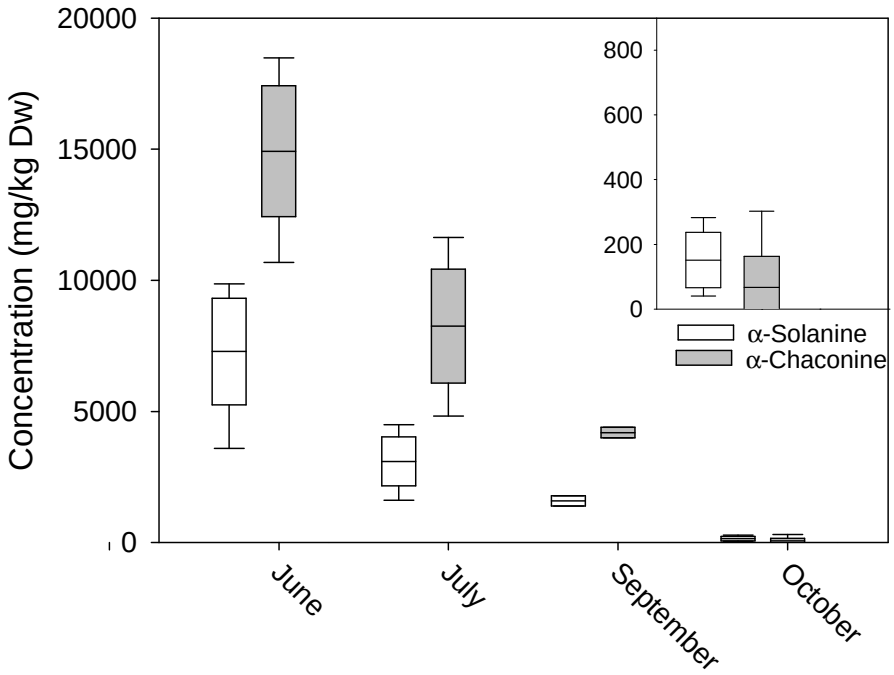

Figure 2.4 Concentration of glycoalkaloids in potato plants during the growth season. Box indicates mean ± standard deviation and the whiskers indicate maximum and minimum concentrations measured. Enlargement of the October concentrations is shown as insert. DW = dry weight. From **Manuscript III**.

To predict a possible mass load of glycoalkaloids from the potato plant to the underlying soil, the total amount rather than the concentration of glycoalkaloids in the plant is of interest. Kolbe and Stephan-Beckmann (1997a; 1997b) presented data for leaves, stems and tubers. They showed that the leaves and stems combined contained the main proportion of the glycoalkaloids in the plant (51-76% depending on the time of the growth season); the leaves being the main source of those two. The maximum amount of glycoalkaloid in the leaves was observed 45-75 days after emergence, which is in accordance with the present study (**Manuscript III**). Here, the highest amount of glycoalkaloids in leaves and stem combined was detected in July, because of the high biomass at that time, even when the highest concentration was found earlier in June. The amount detected in July corresponded to 25 kg/ha. In the study by Kolbe and Stephan-Beckmann (1997a; 1997b), an equal glycoalkaloid distribution between leaves and tubers was observed by the end of the growth season after four months. Most of the tubers will though be removed from the field during harvest, why only the minor fraction of tubers left in the field after harvest may possess a source of glycoalkaloids to the soil.

2.5 Influencing Factors on Production

Many factors affect the glycoalkaloid production in the potato plant. Most knowledge is available about the production in the tubers because of their use for consumption. The content is overall genetically controlled (Friedman and McDonald, 1997). This is illustrated by a study of eight commercial varieties, where the total glycoalkaloid concentration varied from 7.3-187 mg/kg FW in

the tubers (Friedman et al., 2003b). Additionally, the production of glycoalkaloids in the tubers is affected by physiological stresses during growth and after harvest. The possible influences by various stress factors have been investigated in numerous studies, and a review can be found in Friedman and McDonald (1997). Weather conditions are a major stress factor during growth, and especially cold rainy weather may induce glycoalkaloid production. Potato plants may also produce more glycoalkaloids as a response to stress caused by insect attack; e.g. potato plants attacked by Colorado potato beetles (*Leptinotarsa decemlineata*) produced tubers with higher glycoalkaloid content compared to non-attacked plants (Hlywka et al., 1994; Dinkins et al., 2008). After harvest, factors as light, storage conditions, and mechanical injury are determining for the glycoalkaloid production in the tubers (Friedman and McDonald, 1997); higher concentrations are e.g. induced by bruising (Dale et al., 1998) and light (Dale et al., 1993).

2.6 Biological effects

2.6.1 Toxicity

Ingestion of blighted or sprouted potato tubers or of other parts of the potato plant have for many years been known to possible cause poisoning. Further, it is common knowledge that green potato tubers should not be consumed because of their high glycoalkaloid content. Friedman and McDonald (1997) refer several cases of human poisoning described in early literature earlier than 1960. In a commonly cited case, 78 schoolboys became ill after eating lunch (McMillan and Thompson, 1979). The lunch did partly consist of potatoes, which had been left in the kitchen during a long school break. All ill boys had eaten potatoes and analysis of the remaining potatoes showed a high glycoalkaloid content of those. 17 of the boys required hospitalization and common symptoms were nausea, vomiting, diarrhoea, and abdominal pain. In a controlled experiment, volunteers developed nausea and diarrhoea after being served potatoes with high glycoalkaloid content (Hellenäs et al., 1992).

The two main toxic effects of the glycoalkaloids are an inhibition of two cholinesterases and cell disruption (Friedman, 2006). The inhibited cholinesterases are the acetyl- and butyrylcholinesterase who both catalyze the hydrolysis of the neurotransmitter acetylcholine at the synapse in the central nervous system. Equal effect in inhibition of the cholinesterases is shown by both glycoalkaloids, and no synergistic effects between the two have been observed (Roddick, 1989). The aglycone, solanidine, showed no inhibition effect. The inhibition is probably due to a non-covalent competitive binding to the active site of the enzyme (Friedman, 2006). The other main toxic effect, cell disruption, is caused by a complex formation with the cell membrane (Friedman, 2006). The aglycone part of the molecule is inserted into the membrane bilayer, where it can interact with membrane sterols. This can result in a rearrangement of the membrane, whereby the membrane structure is disrupted and the cell content leaked. Especially α-chaconine forms strong complexes with cholesterol and other phytosterols (Roddick, 1979). α-Chaconine is a much more potent cell disrupter than α-solanine, but synergistic effects are observed when they are both present (Friedman and McDonald, 1997; Friedman, 2006).

Several other effects in animals have been reported, including the ability to induce spina bifida, anencephaly, embryotoxicity and teratogenicity (Friedman, 2006). The developmental toxicity of both glycoalkaloids and their hydrolysis products were compared; α-chaconine was reported to be more toxic than α-solanine (Friedman et al., 1991; Rayburn et al., 1994). The hydrolysis product β_2-chaconine was about as potent as α-chaconine, while solanidine and the other β- and γ-compounds were less toxic than their corresponding α-compound (Friedman et al., 1991; Rayburn et al., 1994). Estrogenic effects were not observed for the two glycoalkaloids, while low effect in vitro was observed for solanidine (Friedman et al., 2003a). The potato glycoalkaloids do however also have some beneficial properties; the compounds are reported to inhibit growth of tumor cells from e.g. human colon and liver (Lee et al., 2004; Friedman et al., 2005).

To compare the toxicities of the two glycoalkaloids and their hydrolysis products, all the toxic effects are to be taken into consideration. Overall, Friedman and McDonald (1997) concluded, that α-chaconine is the most potent potato glycoalkaloid, and that the hydrolysis products seem to lose toxicity as they lose the carbohydrate moieties. Solanidine is the least toxic compound.

2.6.2 Interactions with Organisms

The potato glycoalkaloids are considered to be a part of the plant chemical defensive system, but the exact role is still undefined (Roddick, 1996; Friedman and McDonald, 1997). Many studies have investigated the interaction between the glycoalkaloids and different pathogens with variable results.

The effect of glycoalkaloids on bacteria is poorly studied. No correlation was found between the glycoalkaloid content in cultivars and their resistance to neither ring rot caused by *Corynebacterium sepedonicum* nor common scab caused by *Streptomyces scabies*. Hence, it was concluded that the glycoalkaloids were not the factor determining the resistance (Paquin, 1966; Frank et al., 1975). An investigation of the toxicity of α-solanine towards microorganisms showed that cultures of *Bacillus subtilis*, *Micrococcus luteus*, *Erwinia* spp. and *Pseudomonas* spp. were unaffected by a concentration of 2,000 mg α-solanine/L phosphate buffer (McKee, 1959). In another type of study, the glycoalkaloids were proven to have antibiotic properties; mice treated with low doses of glycoalkaloids were resistant towards lethal doses of *Salmonella typhimurium* (Gubarev et al., 1998).

The glycoalkaloids possess a strong in vitro activity against fungi (Roddick, 1987) and growth inhibition of a fungi, *Cladosporium fulvum*, was reported as early as 1933 (Schmidt, 1933). Various studies have investigated the effect of the glycoalkaloids on spore germination and fungal growth. Effect on spore germination has been shown for *Ascobolus crenulatus*, *Phoma medicaginis* and three *Fusarium* species (McKee, 1959; Fewell and Roddick, 1993). Inhibition of growth was observed for four fungi; *Ascobolus crenulatus*, *Alternaria brassicicola*, *Phoma medicaginis*, and the potato pathogen *Rhizoctonia solani* (Fewell and Roddick, 1993; Fewell and Roddick, 1997). Pronounced synergism between the two glycoalkaloids was observed. Also, a pH effect was found; higher toxicity was seen at pH 7 compared to pH 6. The pH effect, which had also been observed in earlier studies (McKee, 1959), was assigned to a higher toxicity of the uncharged form of the

compound. Other potato pathogenic fungi may not be affected by the glycoalkaloids, because they can overcome the toxicity of the glycoalkaloids by converting them into less toxic compounds (e.g. Weltring et al., 1997), a subject which will be discussed further in Chapter 5.

Adverse effects of glycoalkaloids on behavioral and developmental biology are described for several insects (Tingey, 1984; Friedman and McDonald, 1997). E.g. Sanford et al. (1996) described how the mortality increased for potato leafhoppers (*Empoasca fabae*) dieting on a glycoalkaloid containing diet. Similarly, reduced fecundity, diet uptake and increased mortality were observed for peach potato aphids (*Myzus persicae*), which were fed on an artificial diet containing glycoalkaloids (Fragoyiannis et al., 1998). Also, non-insects may be affected by the glycoalkaloids; in a feeding study of snails (*Helix aspersa*) both glycoalkaloids were shown to possess antifeedant activity. Here, α-chaconine was the more active compound, and α-solanine and α-chaconine interacted synergistically (Smith et al., 2001).

3 Determination of Potato Glycoalkaloids

In the following, important factors and requirements for determination of naturally produced organic compounds in plant and soil matrices are discussed with special emphasis on the potato glycoalkaloids. Further, the range of methods previously used for determination of the glycoalkaloids is presented and discussed.

The determination of an analyte, irrespective of origin, in a solid matrix (e.g. soil or plant material) can be divided into three steps. The first step is an extraction of the analyte from the matrix into an appropriate solvent for analysis. Secondly, interfering compounds have to be removed, and finally, the concentration of the analyte has to be determined. Additional steps, such as pre-concentration or derivatization, may, depending on the chosen analytical method, be needed as well. Extraction, clean-up, and analysis methods will be described in detail in the following sections.

3.1 Extraction

Before analysis of a compound in a soil or plant sample can be performed, the compound has to be extracted from the matrix by use of an appropriate solvent. The optimal extraction procedure should extract 100% of the analyte, while the extraction of interfering compounds should be minimal. Any co-extracted interfering compounds may also be removed later during the clean-up.

A simple and commonly used method is the shake extraction, where the matrix and an appropriate extraction solution are mixed in a bottle or tube and shaken for a certain amount of time, after which the extraction solution is removed and prepared for analysis. The huge advantage of this method is the lack of need for any specialized instrument, by which the expenses may often be increased and the number of possible extractions may be limited. Disadvantages may be the sometimes rather labor-intensive procedures and the amount of solvent used. In stead, more sophisticated extraction techniques such as e.g. accelerated solvent extraction (ASE) or Soxhlet extraction can be used. They may often provide a more efficient extraction due to instrumental setup. E.g., by Soxhlet extraction, multiple extractions are done repeatedly by a re-distillation of the solvent and by use of ASE, a combination of raised temperature and high pressure improves the extraction process (Rubinson and Rubinson, 2000). Other advantages include an automation of the extraction process and/or a reduction in the amount of solvent needed. The availability of the instrument and the number of samples extracted simultaneously may though limit the use in practice.

In the present work, shake extractions were performed, because this was used in the initial work, which was based on a standardized method for extraction of potato glycoalkaloids from plant material (AOAC, 2000). Since this method worked well, there was no need for a change to a more sophisticated method with all the additional limitations and complications. Different parameters can be optimized when using the shake method. The choice of extraction solution is the most vital parameter and depends on the compound in question as well as the matrix. The length of extraction and the temperature during the extraction are other important parameters. Time should be long enough to allow full extraction, but as short as possible to avoid possible transformation of the

compound and to ease the work. Similarly, depending on the compound in question, temperature can be raised to ease the extraction or lowered to avoid possible transformation. Further, a second extraction using fresh extractant may, if necessary for a complete extraction, be performed after removal of the first extraction solution.

3.1.1 Extraction from Plant Material

Methods for extraction of xenobiotics are most often developed on samples spiked with pure compound. However, when working with natural compounds, the plant material does already contain an amount of the compound, which may be bound within the plant material. To ease the release of the analyte during the extraction process the plant material is often dried and comminuted prior to extraction. It is impossible to prove, if an extraction of a natural compound is complete, because the original concentration in the plant material is unknown. It is however possible to compare the amount extracted by different extractants. In practice, different extraction procedures and consecutive extractions may be performed to probe the probability of a complete extraction. In addition, ordinary recovery experiments, where pure compound is added to the plant material can be performed too, but the binding of the naturally present and the spiked compound may differ, why their extraction efficiencies may differ as well.

Table 3.1 Solutions tested for glycoalkaloid extraction from tubers by Friedman and McDonald (1995a; 1997). No reports of the obtained extraction efficiencies were given.

	Extraction solution	Acidic	Organic solvent
(1)	2-5% acetic acid	✓	
(2)	0.5% $NaHSO_3$ in 2% acetic acid	✓	
(3)	0.02 M Na-1-heptanesulfonate in 0.17 M acetic acid	✓	
(4)	0.4 % 1-heptanesulfonic acid in 1% acetic acid	✓	
(5)	5% trichloroacetic acid in 50-75% methanol	✓	✓
(6)	Methanol/acetic acid/water (94:1:6)	✓	✓
(7)	Tetrahydrofuran/water/acetonitrile/acetic acid (5:3:2:0.1)	✓	✓
(8)	Chloroform/acetic acid/methanol (10:1:9)	✓	✓
(9)	Methanol/chloroform (2:1)		✓
(10)	Ethanol		✓

As the glycoalkaloids are only sparingly soluble at pH 7 or above, they are usually extracted from plant material by a non-aqueous and/or acidic extraction solution (Friedman and McDonald, 1997). The combination of heat and acid is usually avoided, because this can cause hydrolysis. Exceptions are methods, where the later quantification is based on the aglycone, why previous hydrolysis is required. More than 20 different extraction solutions are described in the literature (Friedman and McDonald, 1997); most of them involve a weak solution of acetic acid and possible an addition of an organic solvent (e.g. methanol, tetrahydrofuran, acetonitrile, or chloroform). Friedman and McDonald (1995a; 1997) tested ten of the previously published solutions on dried, fresh, and processed tubers (**Table 3.1**); the results showed 2% acetic acid to be the best solution for dried samples, whereas fresh samples were better extracted by a mixture of methanol/chloroform or a mixture of tetrahydrofuran/water/acetonitrile/acetic acid. The official AOAC method for

determination of glycoalkaloids in potato tubers prescribe the use of an extraction solution similar to the aqueous acetic acid solution – a solution of water/acetic acid/$NaHSO_3$ (100+5+0.5, v/v/w) (AOAC, 2000). The sodium bisulfite is added to retard oxidation during sample preparation and analysis (Carman et al., 1986). This method was chosen for extraction of the glycoalkaloids from the plant material in the present work. Here, the efficiency of the method was tested by three consecutive extractions on the same freeze dried plant material. It was hereby shown that 86-91% of the glycoalkaloids were extracted during the first extraction, why only one extraction was performed in the work in **Manuscript III**.

3.1.2 Extraction from Soil

In soil, the analyte may be sorbed, why the extraction can be a more complicated task. Sorption can occur to soil material as organic matter or clay. The amount of analyte in soil may roughly be divided into two fractions; a sorbed fraction and a fraction present in the soil solution, and the two fractions are not extracted equally well. For these reasons, it is important to establish which fraction is of interest, before a new extraction method is developed. It can be of interest to perform consecutive extractions of increasing strength to obtain information on the sizes of the different fractions as done in Jensen et al. (2004). In other cases, sufficient information will be obtained by performing one single extraction extracting one or both fractions. Extraction of the potato glycoalkaloids from soil by a mild extractant (aqueous acetic acid) was previously shown to give poor recoveries (Jensen et al., 2007), indicating that only little glycoalkaloids were present in the soil solution. For this reason, stronger extractants were used in the present work by which the sorbed fraction was extracted as well.

When a new method is developed in order to extract a natural compound from soil, an optimized method for plant extraction of the same compound often exists. This work is often used as basis for the further development, and the method can sometimes be directly transferred. However, the two matrices are completely different, and while the natural compound is bound in the plant matrix, sorption to clay or organic matter may take place in the soil. These interactions in the soil may, depending of the compound in question, be stronger, why a stronger extractant is sometimes needed for soil extraction. One example of a compound, where similar extractants have been used for both matrices, is the weakly sorbed compound, ptaquiloside, produced by bracken fern (Rasmussen et al., 2005). In the early work, ptaquiloside was extracted from both plant and soil material using pure water (Rasmussen et al., 2003a; Rasmussen et al., 2003b), and only a slight modification by the addition of a buffer to avoid a possible fast transformation of ptaquiloside was introduced later (Ovesen et al., 2008). Also, thujone, produced by western red cedar (*Thuja plicata*), was extracted from needles as well as soil samples using steam distillation (Strobel et al., 2005). Similarly, an extraction solution previously applied to plant material (70% methanol) was tested and used on soil material for extraction of glucosinolates produced by plants in the family *Brassicaceae* (Gimsing et al., 2005). In another example, different solutions were used to extract the mycotoxin, zearalenone, from soil (Hartmann et al., 2008). For extraction from wheat, a mixture of acetonitrile and water was frequently used, and this is known to give complete extraction. However, in soil higher

extraction efficiency and lower standard deviation were achieved by use of another stronger extractant (100% methanol).

Glycoalkaloids have, as previously described, been extracted from potato plant material by use of many different extraction solutions. A selection of these was also tested during the initial development of an extraction method for soil. In Jensen et al. (2007), four solutions were tested for extraction from soil material (**Table 3.2,** 1-4); solutions 1-3 were previously used for extraction from plant material (Bushway et al., 1980; Bushway et al., 1985; AOAC, 2000). Only one of these extractants (the mixture of tetrahydrofuran/water/acetonitrile/acetic acid) did extract the glycoalkaloids with a reasonable efficiency. As the recovery was relatively low, the solution was still not an optimal choice, why further improvements were required. From that work, it was observed that addition of acid improved the extraction. Furthermore, an organic solvent was required, because no glycoalkaloids were extracted by an aqueous acid solution solely. The extraction method was improved by Pedersen (2007), who tested the extraction efficiencies of aqueous acidic mixtures of each of the three organic solvents; acetonitrile, methanol and isopropanol. This work showed the mixtures with acetonitrile to give the better recoveries (**Table 3.2,** 5-7). The use of 60% acetonitrile, 0.4 M acetic acid gave α-solanine recoveries of 73-88% for six soil matrices, where the higher recoveries were obtained for soils with little sorption capacity, while the lower recoveries were found for the soil with a higher organic content (**Manuscript IV**). This extraction solution is also used in **Manuscript III**, where the recoveries for the sandy soil from Fladerne Bæk ranged from 83-98%.

Table 3.2 Solutions tested for extraction of glycoalkaloids from spiked soils.

	Extraction solution	**No. of soils tested**	**Recovery α-solanine (%)**	**Recovery α-chaconine (%)**	**Reference**
(1)	5% acetic acid	1	0	ND[a]	(Jensen et al., 2007)
(2)	tetrahydrofuran/water/acetonitrile (50:30:20)	1	34	ND[a]	(Jensen et al., 2007)
(3)	tetrahydrofuran/water/acetonitrile/acetic acid (50:30:20:1)	4	47-63	ND[a]	(Jensen et al., 2007)
(4)	tetrahydrofuran	1	0	ND[a]	(Jensen et al., 2007)
(5)	50-80% acetonitrile, 0.2 M acetic acid	1	57-84	48-63	(Pedersen, 2007)
(6)	50-80% isopropanol, 0.2 M acetic acid	1	7-13	20-31	(Pedersen, 2007)
(7)	50-80% methanol, 0.2 M acetic acid	1	7-14	14-43	(Pedersen, 2007)
(8)	60% acetonitrile, 0.2 M acetic acid	6	73-88	ND[a]	**Manuscript IV**
(9)	60% acetonitrile, 0.4 M acetic acid	1	83-94	89-98	This project, partly in **Manuscript III**

[a] ND not determined

3.2 Clean-up

After the extraction, a clean-up step may be needed to eliminate any interfering compounds. A common method for clean-up is solid phase extraction (SPE). SPE is performed by passing the extraction solution containing the analyte over a solid phase that absorbs the analyte from the solution. Matrix components may additionally be sorbed. The extraction solution, now without the analyte, but still containing any non-sorbing matrix components, is discarded. The solid phase is

thereafter washed, whereby weakly sorbed matrix components are removed, before the elution of the analyte takes place. The major part of the matrix components is generally removed during the SPE clean-up, and the process will often result in a pre-concentration of the analyte as well. Different types of solid phases (apolar, polar, or ionic) can be selected depending on the analyte and the matrix (Rubinson and Rubinson, 2000).

SPE has become the most popular method for purification of the glycoalkaloids (Friedman and McDonald, 1997), and the use is discussed further here, but also other methods as precipitation by ammonium hydroxide, partitioning with aqueous Na_2SO_4, or water-saturated butanol can be used for glycoalkaloid purification (Wang et al., 1972; Bushway et al., 1979; Friedman et al., 1994). Various SPE cartridges have successfully been used for pre-concentration and purification of glycoalkaloids; e.g. of 17 tested cartridges, the 15 gave good recoveries for α-solanine (Jensen et al., 2007). This selection of columns included C2-C18, phenyl and CN columns. Good results have also been obtained using a benzenesulphonate cation exchanger and a macro porous copolymer sorbent (Väänänen et al., 2000). The choice of SPE cartridge and the conditions used do not only depend on the compound of interest, but also on the extraction solvent and the matrix components. To obtain proper retention on a reversed phase column, the compounds should be dissolved in an aqueous solution; why an organic extraction solution may have to be diluted or evaporated prior to application as done in Pedersen (2007) and Jensen et al. (2007). The influence of the matrix can be illustrated by the clean-up of glycoalkaloids from samples with low and high levels of lipids (e.g. tubers vs. potato chips). The samples with low lipid content were cleaned up by a C18 column while better results were obtained by use of an amino (NH_2) column for samples with a high amount of lipids (Saito et al., 1990). Hence, whenever a new matrix or extraction solution is introduced, the SPE procedure should be validated to ensure that proper retention and good recovery are still maintained.

3.3 Quantification

A broad range of analytical methods are in use, when analytes extracted from soil or plant matrices are to be quantified. Only methods, which have been used for glycoalkaloid quantification, will be discussed here. During the years, quite many different methods have been used for glycoalkaloid determination; the choice of and change in methods over time reflect the improvement and development in analytical chemistry. The range of methods generally used for quantification of the glycoalkaloids now or in the past is discussed in the following, while the specific methods used in the present work is discussed further in Chapter 4.

3.3.1 Previously Used Methods

A couple of methods were primarily used in the past, these were later more or less replaced when new instruments or improved techniques were introduced. Among these methods are the colorimetric methods, thin layer chromatography (TLC) and gas chromatography (GC). The total amount of glycoalkaloids was initially quantified by various colorimetric methods (Friedman and McDonald, 1997). After the discovery of the second glycoalkaloid, α-chaconine, in 1954, a method

for quantification of the individual glycoalkaloids was needed, and such a method was developed by Paseshnichenko and Guseva (1956) using paper chromatography. TLC and later high performance thin layer chromatography were both frequently used techniques for separation and determination of the individual glycoalkaloids and the hydrolysis products, e.g. (Jellema et al., 1981; Filadelfi and Zitnak, 1983; Ferreira et al., 1993; Friedman et al., 1993; Simonovska and Vovk, 2000; Bodart et al., 2000). TLC is nowadays more or less replaced by high performance liquid chromatography (HPLC), but new applications are still published (e.g. Skarkova et al., 2008). TLC was commonly performed using a silica gel plate as stationary phase and a solvent system, which consisted of variable combinations of chloroform, methanol, ammonia, and water. Several detection methods of variable specificity exist, e.g. antimony trichloride, Dragendorff reagent, anisaldehyde reagent, iodine vapor, Ce(IV)sulfate-sulfuric acid reagent, and optical brighteners (Cadle et al., 1978; Jellema et al., 1981; Filadelfi and Zitnak, 1983; Ferreira et al., 1993; Friedman et al., 1993; Simonovska and Vovk, 2000; Bodart et al., 2000; Skarkova et al., 2008).

GC coupled with a flame ionization detector can also be used for quantification of the glycoalkaloids. The glycoalkaloids may be quantified individually after a permethylation (Herb et al., 1975) or in total when the glycoalkaloids are hydrolyzed to their corresponding aglycone prior to analysis (Lawson et al., 1992). The direct analysis of the aglycones may shorten the column life as high temperatures are required in the GC system, why it may be preferred to perform a derivatization of the aglycones before analysis (Laurila et al., 1999). In this later work, the GC was coupled with a mass spectrometer (MS).

3.3.2 High Performance Liquid Chromatography (HPLC)

HPLC-UV is the dominating method used for separation and determination of potato glycoalkaloids. The first HPLC-UV method was published almost 30 years ago (Bushway et al., 1979), where separation of α-chaconine and α-solanine were obtained by use of a carbohydrate analysis or an amino column. The later development of new and improved techniques, columns etc. have resulted in a range of improved methods (e.g. Carman et al., 1986; Friedman and Levin, 1992; Edwards and Cobb, 1996; Kuronen et al., 1999; Friedman et al., 2003b). Most methods concern separation of the compounds in potato tubers or plants, but also methods for other matrices as processed potato products and blood serum have been developed (Saito et al., 1990; Hellenäs et al., 1992). In general, columns with a C18 stationary phase are the preferred choice, but other stationary phases as C8 and NH_2 can also be used. The glycoalkaloids contain no chromophores and have only little UV absorption. Determination is usually performed by UV detection at 200-215 nm, why the choice of eluent for the separation is limited. Mixtures of acetonitrile/water or acetonitrile/alcohol to which salt, acid pH or both are added in order to improve the separation are commonly used (Friedman and McDonald, 1997). Typical detection limits using HPLC-UV are 2-8 mg/L (Sotelo and Serrano, 2000; Friedman et al., 2003b). The official AOAC method for determination of glycoalkaloids in potato tubers uses a C18 column in combination with an eluent of 60:40 acetonitrile/water (0.01 M phosphate buffer, pH 7.6) (AOAC, 2000). Detection is performed at 202 nm. This method has been used in the present work, and the principles behind HPLC-UV separation and determination are discussed further in Chapter 4.

3.3.3 Mass Spectrometry (MS)

MS has been used alone or in combination with a previous separation method for determination of the glycoalkaloids. Alone, MS has been performed by use of a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) (Abell and Sporns, 1996). The method was compared to the ordinary HPLC-UV determination and it was presented as a fast method with less need for sample clean-up. The major disadvantage was the cost of the instrument. MALDI-TOF was later used by the same research group for analysis of glycoalkaloids in potato tubers and in serum (Driedger and Sporns, 1999; Driedger and Sporns, 2001), but the instrument has gained no further use within glycoalkaloid determination.

A few methods using capillary electrophoresis-MS (CE-MS) or GC-MS have been published (Laurila et al., 1999; Bianco et al., 2002), and a detection limit of 10-50 µg/L has been reported for CE-MS (Bianco et al., 2002). However, since HPLC-UV has been the preferred method for many years, the MS has primarily been introduced in glycoalkaloid determination as a replacement for the UV detector. The first method using LC-MS for quantitative determination of potato glycoalkaloids in transgenic potato plants was published in 2003 (Stobiecki et al., 2003). Hereafter, several other methods for quantification of the glycoalkaloids in plants or tubers have been published with detection limits in the range from 0.25 to 39 µg/L (Matsuda et al., 2004; Chuda et al., 2004; Zywicki et al., 2005; Väänänen et al., 2005). The change from UV to MS detection typically lowers the limit of detection, which is also seen for the glycoalkaloids. However, the developed LC-MS methods were developed for analysis of potato tissues, where the glycoalkaloid concentrations are relatively high. Hence, there was no need to optimize the methods in order to obtain a low detection limit.

3.3.4 Other Methods

Several other methods have been used for quantification of the glycoalkaloids. Most of these methods have been developed in order to provide the plant breeders, harvesters and the potato industry with a cheap, easy, and fast method for quantification of the total glycoalkaloid content. One method measured the liposome lysis, which is proportional to the glycoalkaloid content (Bacigalupo et al., 2004). Another method is based on the inhibition of the cholinesterases by the glycoalkaloids (Arkhypova et al., 2003), while more methods using different formats of immunoassays have been presented as well (Friedman et al., 1998a; Glorio-Paulet and Durst, 2000).

3.3.5 Choice of Method

A broad range of methods for quantification have been presented, of which many are still in use. In order to choose a method, first it should be clarified if a quantification of the individual glycoalkaloids is needed or if only the total amount is of interest. Furthermore, the expected concentration range of the glycoalkaloids is an important factor for the choice. In the potato plant, the concentrations are relatively high, why they can be analyzed by most methods. For the environmental samples, low concentrations are expected, why a more sensitive detection method as

MS is preferred. Practical issues as number of samples and availability of analytical instruments may influence the choice as well.

In the present work, a method for determination of individual glycoalkaloids in environmental samples (soil and groundwater) was needed. Hence, a sensitive method as LC-MS was the preferred choice. Only few LC-MS methods were published back then and they had little focus on the obtained detection limit, so it was decided to develop a quantitative LC-MS method. The principles behind LC-MS are discussed in more details in Chapter 4, and the developed method is published in **Manuscript I**.

4 Analytical Methods Used in This Project

4.1 High Performance Liquid Chromatography (HPLC)

HPLC is one of the most commonly used techniques for separation of non-volatile organic components. In chromatography in general, compounds are selectively distributed between two immiscible phases. In HPLC, the phases consist of a mobile liquid phase and a stationary phase packed in a column. The mobile phase is forced through the column by high pressure and the sample components are continuously distributed between the two phases. The separation proceeds according to the compounds affinities for each of the two phases. The components are eluted from the column at a specific time for each component, whereby the desired analytes can be detected individually. Separation can be performed by either isocratic or gradient elution. By an isocratic elution, the mobile phase is kept constant for the entire separation. In contrast, the mobile phase is gradually changed towards a more desorbing eluent in gradient elution. Gradient elution is often needed if a range of structural different analytes are to be separated and analyzed in order to obtain separation and elution within a reasonable time frame (Rubinson and Rubinson, 2000).

The choice of mobile and stationary phases depends on the sample type, the analytes, and the detection method. A number of stationary phases are available and they interact with the analytes by different mechanisms. The HPLC separations are categorized based on the type of interaction. The dominant type of HPLC separation today is reverse phase separation, where a non-polar stationary phase is used in combination with a more polar mobile phase. The predominant stationary phase in reverse phase HPLC consists of non-polar groups as $-C_{18}H_{37}$ bonded onto silica. This stationary phase is known as C18. Shorter apolar chains as e.g. $-C_8H_{17}$ may also be used. Mobile phases in reverse phase chromatography are mixtures of water and an organic solvent such as acetonitrile or methanol to which a buffer can be added. The choice of mobile phase can be restricted by the choice of detector, as e.g. some mobile phases have a high UV cut-off while other are incompatible with MS detection due to e.g. low volatilization. Other types of HPLC separations include normal phase, ion exchange, and size exclusion chromatography (Rubinson and Rubinson, 2000). In the present project, reverse phase chromatography has been used throughout the work.

4.2 Detectors

A range of detection systems can be coupled with an HPLC. Fluorescence, UV, and MS detectors are all commonly used. The choice of detector is largely dependent on the properties of the analytes in question, as e.g. only fluorescent analytes can be detected by a fluorescence detector (Rubinson and Rubinson, 2000). Two type of detectors; UV and MS have been used in this project, why only the use of these detectors will be discussed in further detail here.

4.2.1 UV Detection

UV detection is based on determination of the absorbance at a certain wavelength. The method requires that the analytes in question absorb UV light, why the method is limited to UV light absorbing compounds as aromatics or compounds with conjugated double bonds. The detection

method has a low specificity as all light absorbing compounds at the desired wavelength and with a similar retention time will be misidentified as the analyte. Coupled to the HPLC system, determination by UV can either be performed at a single wavelength, or if a diode array detector is present at several wavelengths at the same time. The latter increases the specificity of the method as more wavelengths or a whole spectrum can be recorded and used for identification.

4.2.2 MS Detection

The MS technique has found use within many fields of research and can be used alone or in combination with several separation techniques. Here, the focus will be kept on the use of MS as a quantitative detector coupled to HPLC. After the effluent has left the HPLC, the first step in MS is an evaporation of solvent, after which the analytes are converted into gas phase molecules and ionized in the ion source. The ions are hereafter transported into the high vacuum of the mass analyzer, where they are separated in space based on mass to charge ratios (*m/z*). For this, a number of different mass analyzers exist and the ones used in this project are presented later. The last region is the transducer or detector, where the signals from the separated ions are amplified; hereby producing a mass spectrum (Rubinson and Rubinson, 2000; Dass, 2007).

Electrospray Ionization

MS analysis requires high vacuum conditions, and before the analytes reaches the mass analyzer, an interface is needed to eliminate the large volume of material in the effluent from the HPLC. The interface electrospray ionization (ESI) has become the most successful interface for LC-MS and it operates at atmospheric pressure. In ESI, the mobile phase is led into the source through a stainless steel capillary. A high voltage is applied to the tip of the capillary, whereby a so-called Taylor cone with an aerosol of electrically charged droplets is formed. A flow of hot nitrogen assists and stabilizes the nebulization process and the evaporation of the eluent. The size of the droplets is reduced by the evaporation of the eluent and finally the charge density of the droplets becomes too high and the repulsion too great and the droplets fall apart. The final result is a formation of free ions in gas phase, after which the ions can be transported into the vacuum. It is a soft technique compared to other ionization techniques, and only little fragmentation of the compounds occurs (Bruins, 1998; Dass, 2007). It is though possible to induce fragmentation by increasing the sampling cone voltage, a technique which can be of use in compound identification (Dass, 2007).

The ionization by the ESI may be carried out in either positive (ESI+) or negative (ESI-) mode depending on the analytes. The effectiveness of the ionization process is affected by the pH and the solvent (Bruins, 1998; Rubinson and Rubinson, 2000). High surface tension and viscosity hamper the production of the droplets, why highly aqueous mobile phases are not favorable. Not all buffers are compatible with the use of ESI, non-volatile buffers as e.g. phosphates, should be avoided, because they may precipitate in the mass spectrometer and thereby disturbing the analysis (Niessen, 2006). The effectiveness may also be affected by the matrix, which often tends to reduce the ionization (Stüber and Reemtsma, 2004).

Mass Analyzers

From the ESI, the ions are transported into the high vacuum of the mass analyzer, where they are separated in space based on *m/z*. A number of different mass analyzers exist. The types used in this project – the quadrupole and the time-of-flight (TOF) mass analyzers – will be presented here.

The mass analyzer in a TOF instrument consists of a field-free flight tube, where the ions are separated according to their travel time to the detector. The ions travel with different velocities depending on *m/z*, the smaller *m/z* being the fastest. When the ion beam enters the mass analyzer, the beam is pulsed towards the reflectron in the end of the flight tube. The reflectron is an ion-mirror which minimizes the dispersion of the ions of same *m/z* and reflects the beam back. The travel path has a form as a "V" in a MS with one reflectron (**Figure 4.1**). Newer instruments may have two reflectrons, whereby the mass resolution is increased; here the travel path has a form as a "W" (**Figure 4.1**). The *m/z* values of the ions are determined from their travel time, and a mass spectrum is produced. A complete mass spectrum can be produced every 25 µs (Dass, 2007).

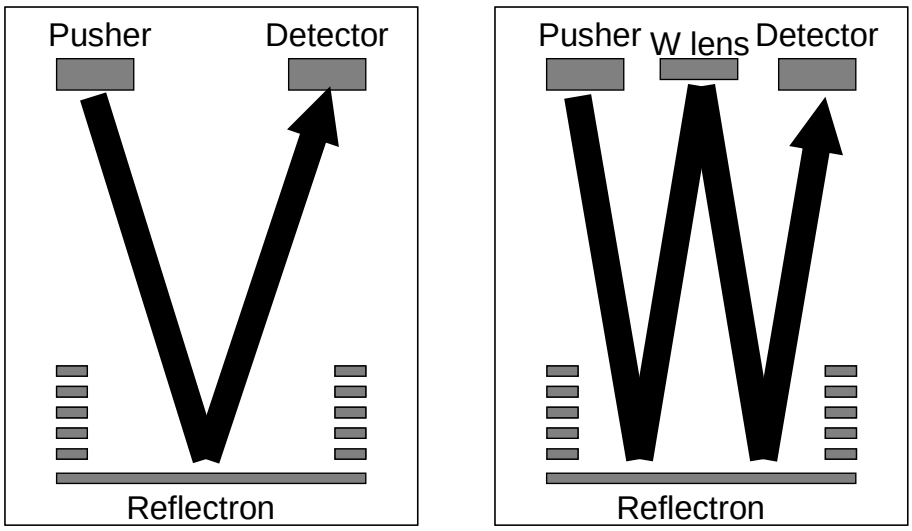

Figure 4.1 Principle for TOF mass analyzer in "V" and "W" mode.

TOF instruments work within an almost unlimited mass range or in practice up to *m/z* 10,000-20,000. The ability to produce a complete mass spectrum within short intervals is a major advantage for this type of mass spectrometers. The obtainable mass resolution is high; up to 10,000 (full-width at half maximum (FWHM)). By additional use of an internal standard accurate mass determination within ±5 mDa is possible for compounds up to 1000 Da (Niessen, 2006; Dass, 2007). In the past, TOF instruments have primarily been applied for structure elucidation and exact mass measurements. Due to performance improvements of the TOF instruments, quantitative measurements are now also possible, and several methods for quantification are published (e.g. Tolonen and Uusitalo, 2004; Ferrer et al., 2005; García-Reyes et al., 2006; Wang et al., 2006, **Manuscript I**).

A quadrupole instrument consists of four parallel metal rods. An electric field is created by applying direct current and radio-frequency potentials to the rods, whereby the collimated ion beam from the source passes through the center of the array of rods in a complex oscillating path. By applying a defined set of potentials to the rods, only ions within a narrow *m/z* range pass through the rods to the detector, while all other ions collide with the rods and are neutralized. A full mass spectrum can be obtained by varying the applied potentials. Often, the potential is fixed or jumps between a few selected potentials hereby recording only a few *m/z* values. This mode is named selected ion monitoring (SIM). For quantitative purposes, this is preferred, because higher sensitivity will be obtained due to the longer recording time for each specific ion trace (Rubinson and Rubinson, 2000; Dass, 2007). Quadrupole instruments have a practical *m/z* upper limit of 4,000, but the primary use is for analysis of low-mass compounds. The resolution is lower than for TOF instruments; in practice about unit mass. Quadrupoles are among the most widely used types of MS (Dass, 2007).

In environmental quantitative analysis, the use of tandem mass spectrometry is common as well, and in the present work a triple quadrupole MS was also tested. In tandem MS, two or more mass spectrometers are coupled, whereby additional structure-specific information can be obtained. The basic principle in tandem MS is a mass selection of the desired target ion in the first MS. This precursor ion is then fragmentized in a second MS or in a collision cell, after which the last MS can be used for either mass scanning of the product ions or for recording of specific product ions. The latter will be the case in quantitative measurements and is called selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) (Dass, 2007). The use of MRM will improve the selectivity of the method as both precursor and product ion has to meet the selection criteria in each MS.

Matrix Effects

The term matrix effect is defined as an (unexpected) suppression or enhancement of the analyte response due to co-eluting matrix constituents (Niessen, 2006). In order to perform a quantitative analysis, possible matrix effects should be investigated during method development and, if present, dealt with. In MS, matrix effects will most often be due to ion suppression and depends on both the analyte and the matrix (Antignac et al., 2005; Niessen, 2006). Different mechanisms are proposed to explain the ion suppression (Antignac et al., 2005); the most important mechanism is decreased evaporation efficiency due to the presence of matrix components. Other possible mechanisms include co-precipitation of the analytes with matrix components and ionization competition between the analytes and the interfering compounds. There are in general two approaches to deal with an observed matrix effect; either eliminating the interfering matrix components or changing the conditions of the measurement to eliminate the interference (Antignac et al., 2005; Niessen, 2006). The elimination of the interfering components can be done by improved sample clean-up or by improved HPLC separation; both approaches may be time consuming. The influence of the effect can be eliminated by changing the ionization conditions; e.g. changing the ionization mode or use of different mobile phase additives. The use of an internal standard can also eliminate the influence of a matrix effect if the analyte and the internal standard are affected by the matrix in similar ways. Ideally, isotopically labeled internal standards are preferred. The availability of labeled natural

compounds is however rather limited in practice, because the compounds are often not synthesized commercially. The use of matrix-matched standards is another possibility, hereby having similar matrix effects in standards and samples. This may though be cumbersome if samples with different matrices are to be analyzed. The availability of blank matrix solutions might also be limited, when working with natural compounds; e.g. it would not be possible to obtain a potato plant extract without glycoalkaloids.

4.3 Practical Use and Comparison of Implemented Methods

In this project, three different detector systems have been used for quantification of the glycoalkaloids after a previous separation by HPLC: UV, TOF-MS, and single quadrupole-MS. Their performances, advantages and limitations in relation to the determination of the glycoalkaloids in various matrices are discussed below, based on the experiences obtained in this project.

The HPLC-UV method was already implemented in our laboratory (Harder, 2004), but because the HPLC eluent was incompatible with MS detection, a new HPLC method had to be developed. The two HPLC methods are presented in **Table 4.1**. In addition to the exchange of buffer, also the elution mode was changed from isocratic to gradient. By use of a gradient method, solanidine was eluted from the column within a reasonable time and could be included in the method, too.

Further, optimization of the two MS detectors was performed. The TOF instrument was previously mainly used for structure elucidation and compound identification, but now quantitative work can also be performed, and a method for quantitative determination of the glycoalkaloids have been developed; the work is presented in **Manuscript I.** Quadrupole-MS or tandem MS have for the last decade been the preferred choices when quantifying contaminants in the environment by LC–MS (Benotti et al., 2003; Sancho et al., 2006; Gros et al., 2006). In this project, a tandem MS (triple quadrupole MS) was introduced as a substitute for the TOF instrument for practical reasons. During the initial work on the triple quadrupole, no fragments were obtainable from the precursor ions of the glycoalkaloids and solanidine, even when the fragmentation patterns were previously known from the TOF instrument. Hence, it was chosen to use the instrument as a single quadrupole, where quantification was performed using the protonated molecular ions $[M+H]^+$ of α-solanine, α-chaconine, and solanidine.

In **Table 4.1,** the detection limits for the three detection methods are presented. The detection limit for α-solanine was lowered about a factor of 100 and 1,000, when the UV detector was substituted by a TOF-MS or a single quadrupole-MS, respectively. Hence by changing to a MS detector, it was possible to obtain a lower working range, which was needed to perform low concentration degradation studies and to analyze groundwater samples.

Table 4.1 The three detector systems used for quantification of the glycoalkaloids.

Detection method	HPLC method				Detection limit				Ref.
	Eluent	Type	Buffer	Time	Inj. Vol.	α-solanine	α-chaconine	Solanidine	
				Min	µL	µg/L	µg/L	µg/L	
UV	Acetonitrile-water	Isocratic	Phosphate buffer	25	30	410	ND[a]	NI[b]	**Man. IV**
TOF-MS	Acetonitrile-water	Gradient	Ammonium acetate	28.5	10	4.7	2.2	2.5	**Man. I**
Single quadrupole-MS	Acetonitrile-water	Gradient	Ammonium acetate	28.5	10	0.53	0.52	0.12	**Man. III**

[a] ND not determined

[b] NI not included in the method

The development of the new HPLC method was performed using the TOF instrument. By adjusting the settings of this instrument, in-source fragmentation can be induced and information about the fragmentation patterns of the compounds revealed, when full mass spectra are recorded. The fragmentation patterns of the glycoalkaloids and solanidine were presented in **Manuscript I** and are shown in **Figure 4.2**. From α-solanine, fragments of *m/z* 722.5, 706.5, 560.4, and 398.3 corresponding to $[M+H\text{-}Rham]^+$, $[M+H\text{-}Glu]^+$, $[M+H\text{-}Glu\text{-}Rham]^+$, and $[M+H\text{-}Glu\text{-}Rham\text{-}Gal]^+$ were produced in addition to the protonated molecular ion $[M+H]^+$ at *m/z* 868.6 (**Figure 4.2.A**). Similar fragments, where one or more carbohydrate moieties were cleaved off, were produced for α-chaconine (**Figure 4.2.B**). In contrast, no significant fragments were observed for solanidine (**Figure 4.2.C**).

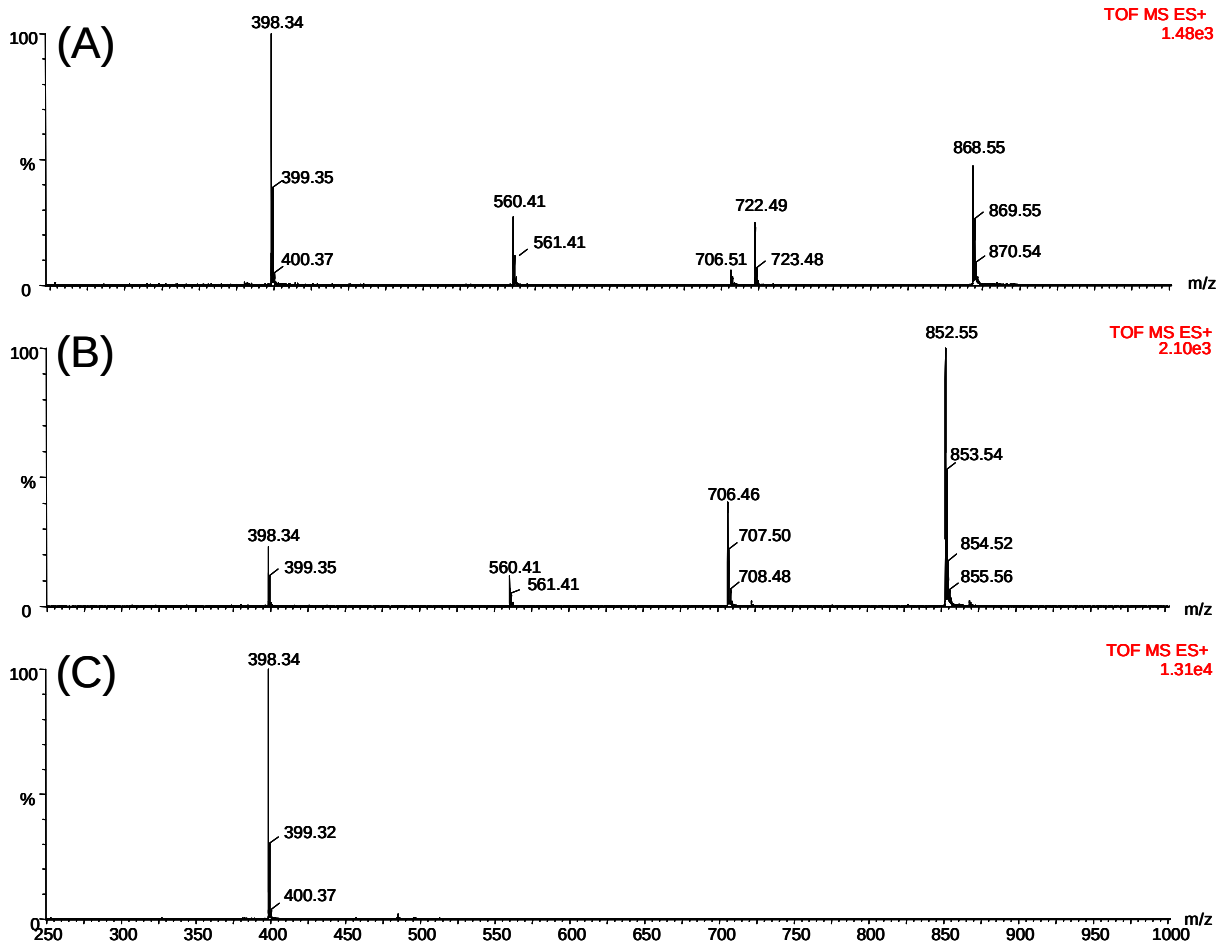

Figure 4.2 Fragmentation patterns for α-solanine (A), α-chaconine (B), and solanidine (C) obtained by TOF-MS using a cone voltage of 70 V and a short distance between the capillary and the cone. From **Manuscript I**.

Matrix effect was investigated for all three instruments by comparing slopes of calibration curves using solvent based standards with slopes from relevant matrix based standards. Only for the single quadrupole, significant matrix suppression was observed. Here, the matrix effect was evaluated by comparing the slopes for standard curves prepared in 20% acetonitrile, groundwater and groundwater amended with 0.02% sodium azide. Suppression of 20-30% was observed for both glycoalkaloids and their aglycone (**Figure 4.3**). To eliminate the influence of the matrix effect, matrix matched standards were used in the later work on the instrument.

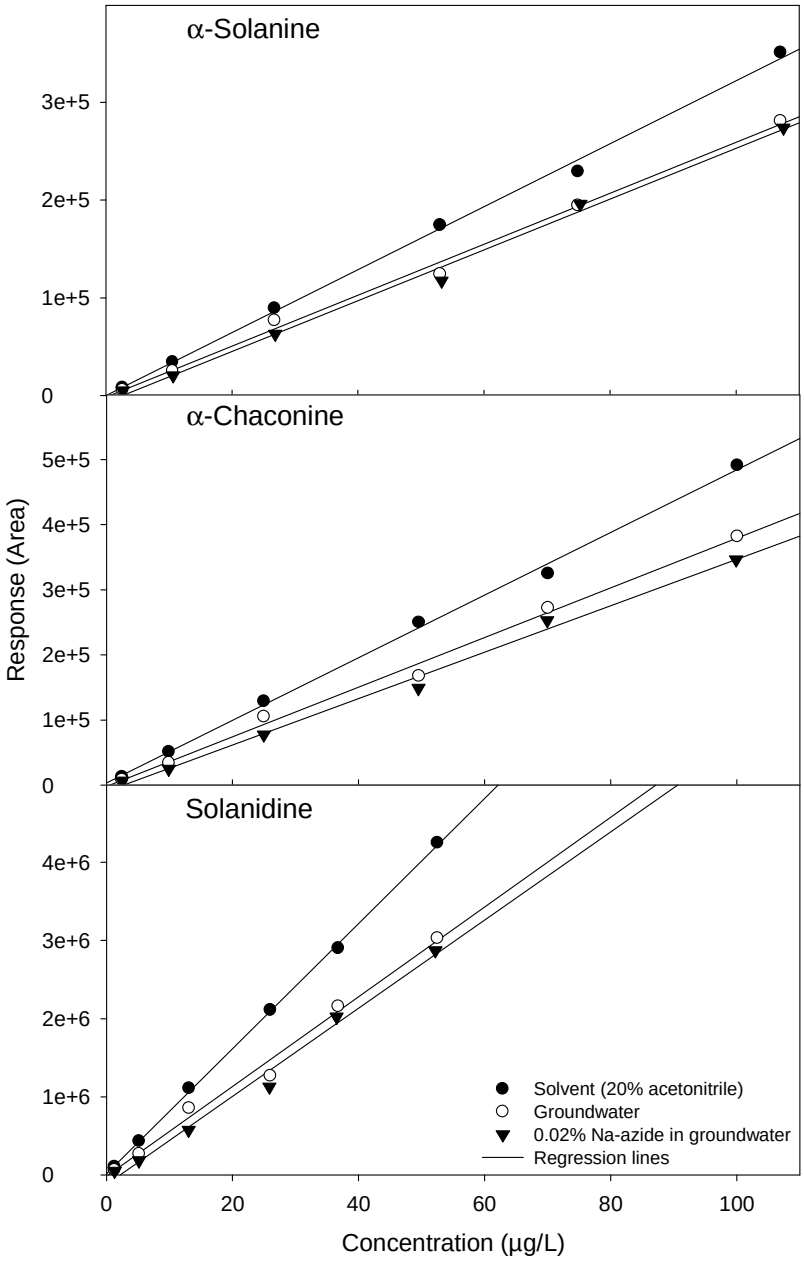

Figure 4.3 Matrix suppression of α-solanine, α-chaconine, and solanidine using LC-single quadrupole-MS. Standard curves obtained for standards in solvent (20% acetonitrile), groundwater and groundwater amended with 0.02% Na-azide are shown. Groundwater was sampled from Fladerne Bæk.

To illustrate the performances and information obtained by the different detection systems, chromatograms from one soil extract, cleaned up by solid phase extraction and analyzed by all three instruments are presented in **Figure 4.4-4.6**. For the UV chromatogram (**Figure 4.4**), rather small peaks of α-solanine and α-chaconine are present, and the shapes of the peaks are a little irregular. In addition, a huge injection peak troubles the quantification, because of the overlap in time with the analytes. A third peak is present in the chromatogram, but no identification is possible by the UV detection.

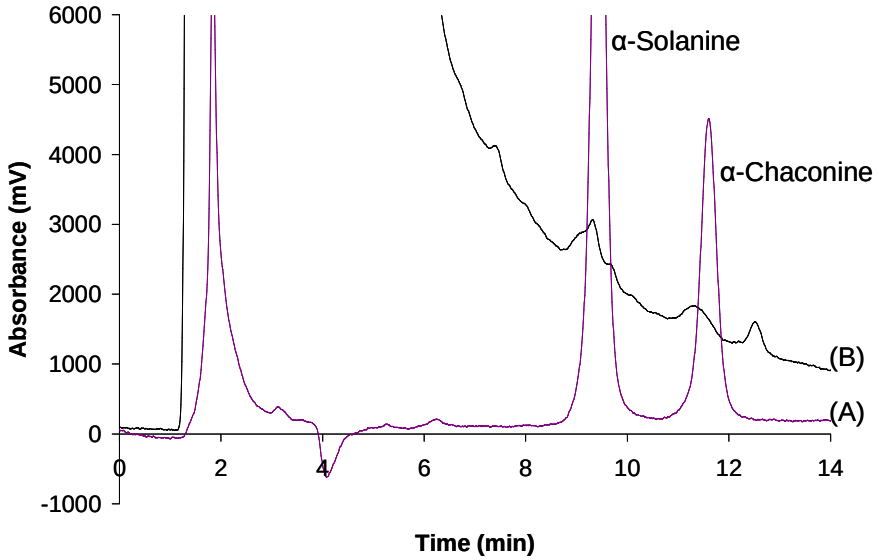

Figure 4.4 HPLC-UV chromatogram for a standard (A) and a SPE cleaned-up soil extract (B). Detection was performed at 202 nm. The sample was run using the following chromatographic conditions; flow 1 mL/min, and isocratic elution using a mobile phase 55% acetonitrile in 0.01M phosphate buffer (K_2HPO_4 and KH_2PO_4, pH 7.6). Further detail about the chromatographic conditions can be found in **Manuscript III**.

More information is obtained by the analysis on the TOF instrument (**Figure 4.5**). First of all, the glycoalkaloid peaks are much more distinct, due to the lower working range of the instrument. Hereby, the identification from the UV chromatogram was confirmed. Secondly, at least one additional peak at 9.4 min with *m/z* 706 is present; this *m/z* value corresponds to the values of β_1-solanine and β-chaconine. Distinction between degradation products with same *m/z* values is not possible in this run, but would be possible if the sample was compared to a sample of degradation products from α-solanine or α-chaconine, solely. No other significant peaks are observed. Any peaks with same retention time as α-solanine or α-chaconine may origin from in-source fragmentation; e.g. the *m/z* 398.3 at 7.07 min is an in-source fragment of α-solanine. Solanidine do also have an *m/z* 398.3 but have a higher retention time (14.0 min).

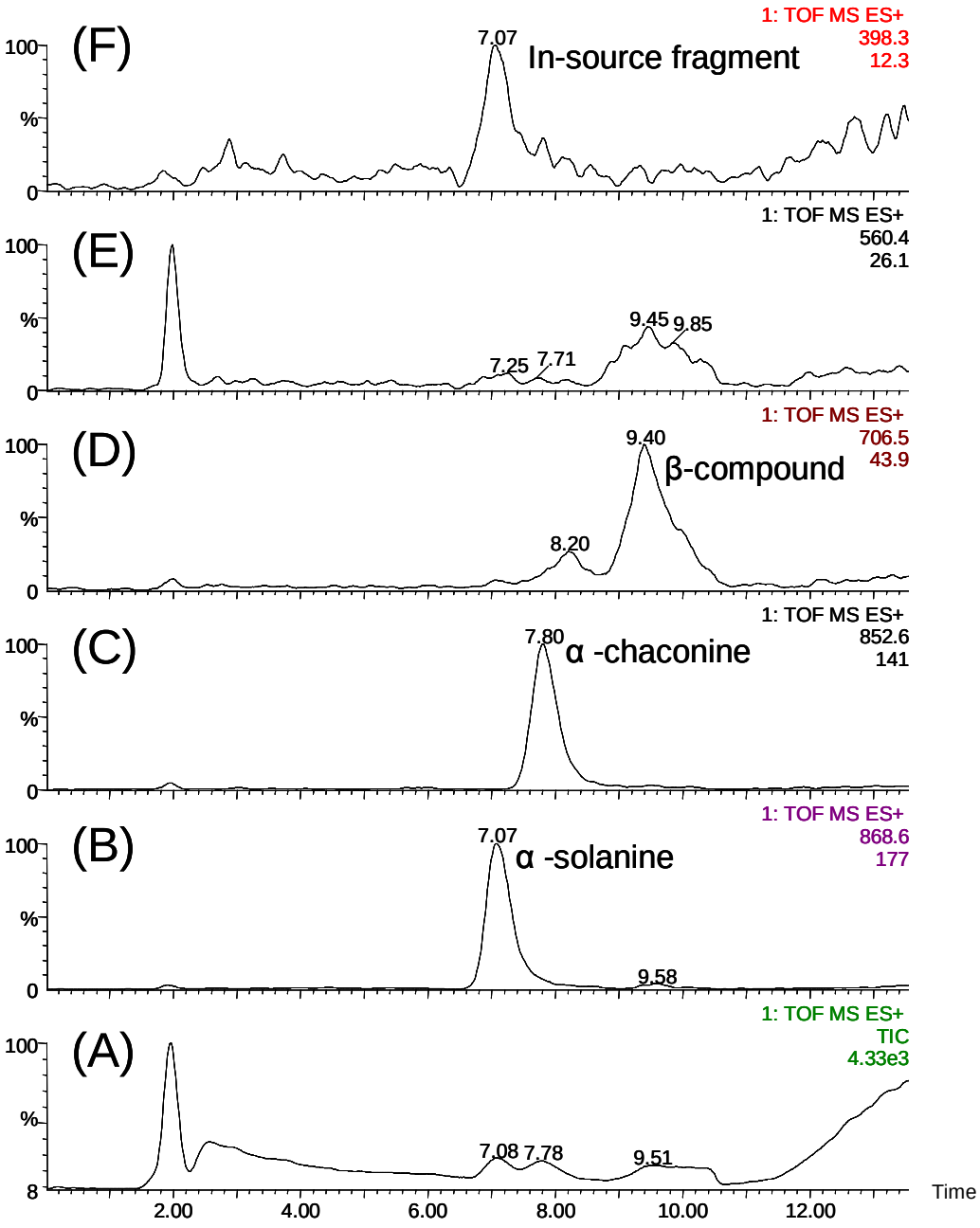

Figure 4.5 HPLC-TOF-MS chromatogram for a SPE cleaned-up soil extract. The sample was run using the chromatographic conditions reported in **Manuscript I**; flow 200 µL/min, and a mobile phase consisting of eluent X (95% water, 5% acetonitrile, 3mM ammonium acetate) and eluent Y (5% water, 95% acetonitrile, 3mM ammonium acetate). The following gradient was applied: 0–6.5 min, 24% Y; 6.5–11.5 min, linear gradient to 80% Y; 11.5–21.5 min, 80% Y; 21.5–24.5 min, linear gradient to 24% Y. (A) Total ion count, (B) *m/z* 868.6 (α-solanine), (C) *m/z* 852.6 (α-chaconine), (D) *m/z* 706.5 (possibly β_1-solanine or β-chaconine), (E) *m/z* 560.4, (F) *m/z* 398.3 (in-source fragment).

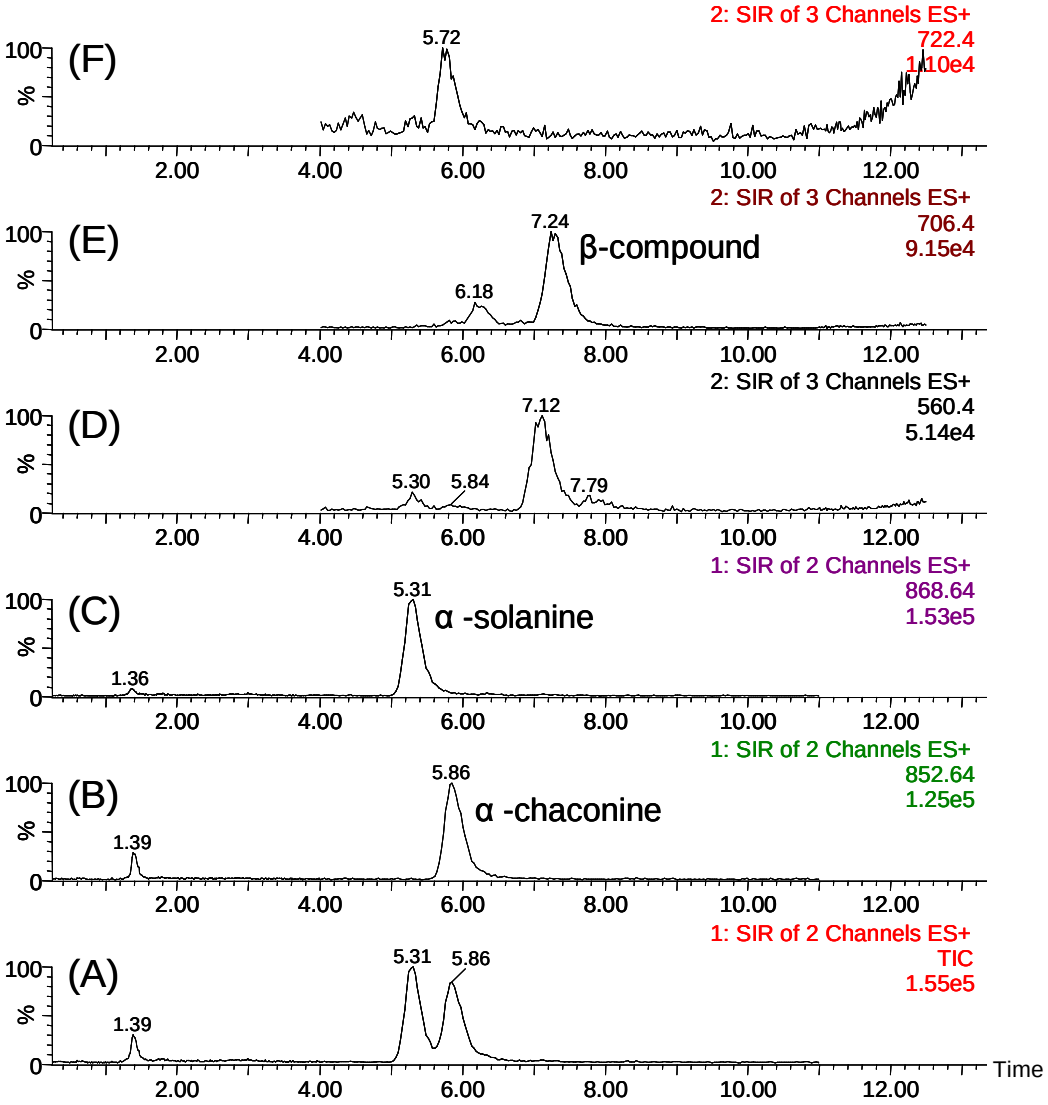

Figure 4.6 HPLC-single quadrupole-MS chromatogram for a SPE cleaned-up soil extract. The sample was run using the MS conditions reported in **Manuscript II**, including the following chromatographic conditions; flow 200 µL/min, and a mobile phase consisting of eluent X (95% water, 5% acetonitrile, 3mM ammonium acetate) and eluent Y (5% water, 95% acetonitrile, 3mM ammonium acetate). The following gradient was applied: 0–6.5 min, 24% Y; 6.5–11.5 min, linear gradient to 80% Y; 11.5–21.5 min, 80% Y; 21.5–24.5 min, linear gradient to 24% Y. (A) Total ion count, (B) *m/z* 852.64 (α-chaconine), (C) *m/z* 868.64 (α-solanine), (D) *m/z* 560.4, (E) *m/z* 706.4 (possibly β_1-solanine or β-chaconine), (F) *m/z* 722.4.

Similar information is obtained by the chromatogram obtained by the single quadrupole instrument (**Figure 4.6**). Distinct peaks are observed for α-solanine and α-chaconine, while also a significant peak is observed at *m/z* 706.4, which may possibly origin from β_1-solanine or β-chaconine. Smaller peaks are also observed at *m/z* 722.4 and 560.4, which correspond to the *m/z* values of β_2-solanine (722.4), γ-solanine (560.4), and γ-chaconine (560.4). Because of their closeness in time to other peaks and their small size, it can not, based on this chromatogram, be excluded that the peaks may originate from in-source fragmentation.

Generally, the soil samples were analyzed only by HPLC-UV, and only α-solanine and α-chaconine were quantified and reported in **Manuscript III**. The chromatograms obtained by mass spectrometry show that at least a third significant peak at *m/z* 706 was present. This was not included in the manuscript, as α-solanine and α-chaconine were the overall dominating glycoalkaloids in the soil extracts, while other peaks were rarely detected and smaller in size. In addition, the extraction and clean-up method for the soil extracts was only developed and optimized for the two main glycoalkaloids, and lower extraction efficiency and overall recovery were expected for any degradation products.

Overall, all three instruments have been used in this project. The UV detection was used in **Manuscript IV,** where the degradation of a relatively high concentration of α-solanine was followed in soil. Plant and soil samples from the field were also analyzed by use of UV detection (**Manuscript III**), because their content of glycoalkaloids were sufficient for UV detection. The UV working range was insufficient for analysis of the groundwater samples (**Manuscript III**), which was instead analyzed by the single quadrupole-MS. By use of the TOF instrument additional detection of non-target compounds was possible. This was used in the preliminary work for **Manuscript II**, where a soil solution with glycoalkaloids was screened for degradation products. Further work for **Manuscript II**, where the degradation of the glycoalkaloids and the formation and degradation of known degradation products were followed, was performed using the single quadrupole instrument. The identification ability by the TOF instrument was additionally used in **Manuscript III and IV**, where unknown peaks in the UV chromatograms were identified.

5 Fate of Glycoalkaloids in the Environment

The fate of an organic compound in the terrestrial environment is determined by processes as sorption, degradation, and leaching. In order to predict the fate a range of studies is needed. In the laboratory, the processes are investigated under controlled conditions, whereby the individual processes can be studied. Thereby, the mechanisms behind can be elucidated and possible degradation products can be identified. The processes influence each other and by use of field studies, information about the fate under realistic but also complex conditions can be gained. Both types of studies are valuable and they provide information about different aspects of fate.

Fate studies have been published for a range of natural bioactive compounds; though, overall the number of studies is relatively small. The group of compounds investigated is diverse in terms of chemical structure and origin (**Figure 5.1**), and the motivation and objectives for the studies differ. Many of the compounds origin from important crop species (DIBOA, DIMBOA, dhurrin, linamarin, lotaustralin, mycotoxins), other compounds are intentionally spread to suppress pests (strychnine and glucosinolates), while a third group of compounds is studied in order to prove and understand their allelopathic properties (juglone and batatasin-III). The potato glycoalkaloids belong to the first group of compounds, as they are produced by an important crop species. In the following, the different type of fate studies will be discussed, and the work on the potato glycoalkaloids will be related to the work published on other natural compounds.

5.1 Degradation

Degradation of organic compounds may be a consequence of either abiotic or biotic processes; including abiotic hydrolysis, microbial degradation or degradation due to plant enzymes. The ultimate degradation, also named mineralization, of an organic compound is primarily due to microbial activity and leads to the formation of inorganic salts and CO_2 (Alexander, 1981). Incomplete degradation leads to the formation of one or several metabolites, which are often of major importance to fate studies, because they have other properties than the original compound. The metabolites may e.g. be more persistent or more polar compounds and hence have a higher risk of leaching (Boxall et al., 2004), but sometimes the opposite is the case as well; e.g. the degradation product of ptaquiloside, pterosin B, is less polar than ptaquiloside (Rasmussen et al., 2005). Hence, knowledge of the degradation pathway is extremely important, when the fate of a compound is being investigated.

In the following, the degradation of the potato glycoalkaloids and the known metabolites will be discussed. In addition to the processes discussed here, the potato glycoalkaloids undergo hydrolysis under acidic conditions. This process was described in Chapter 2, because it was found to be without relevance in the terrestrial environment.

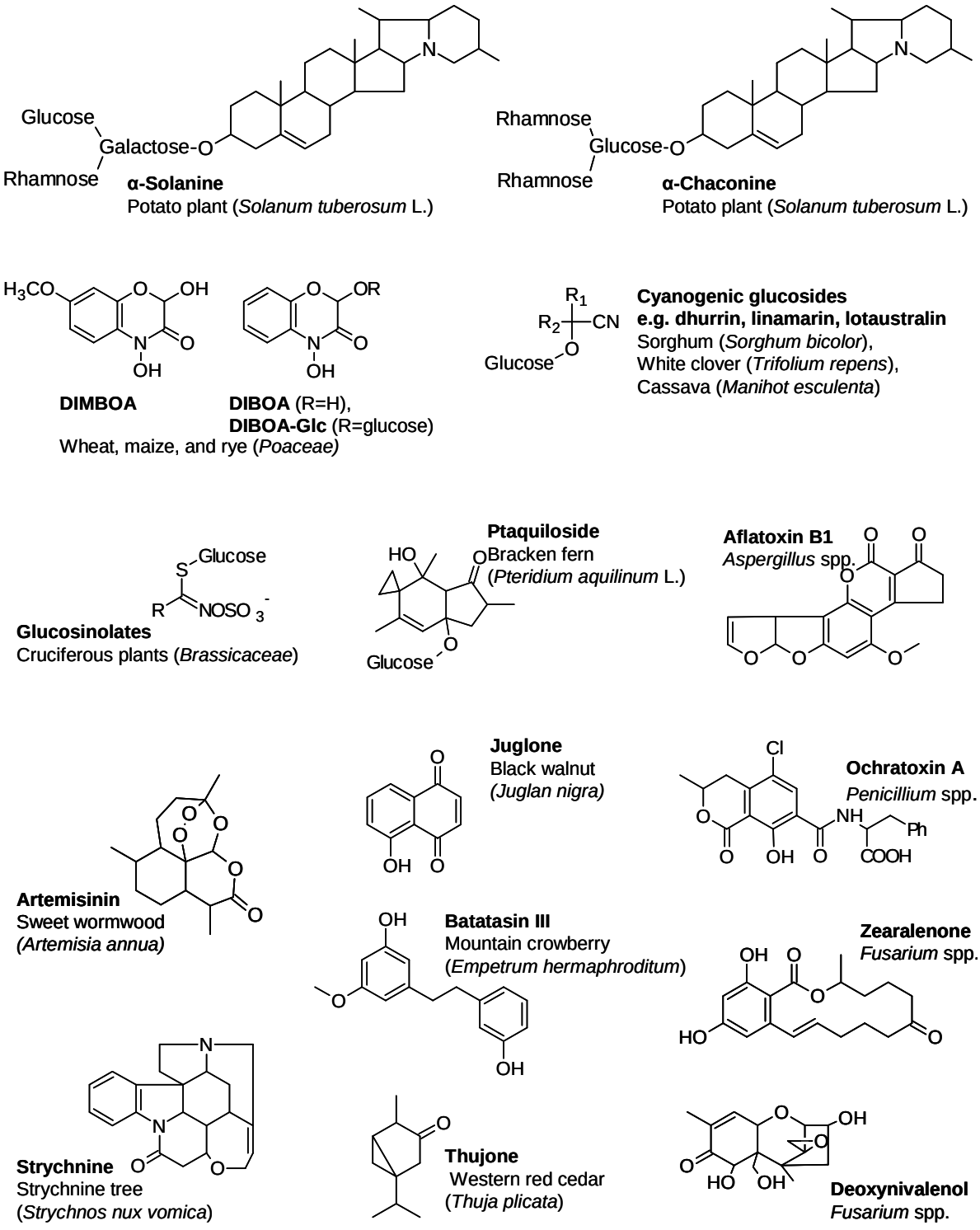

Figure 5.1 Natural compounds investigated in fate studies including the name of the producing plant or microorganism.

5.1.1 Plant Enzymatic Degradation

Plants often produce enzymes to degrade or transform their own bioactive compounds; e.g. the glucose moiety in both glucosinolates and cyanogenic glucosides can be cleaved by enzymes in the plants (Gimsing et al., 2005; Johansen et al., 2007; Bjarnholt et al., 2008). The enzymes and the compounds in these plants do though only get into contact upon tissue damage. Potato plants also contain enzymes, which can hydrolyze the glycoalkaloids. Sprouts, tubers, leaves, berries, and blossoms do all contain enzymes, which hydrolyze the glycoalkaloids. This process leads to the formation of the corresponding β- or γ-compounds or solanidine (**Figure 2.2**). The observed hydrolysis products from the various tissue enzymes are presented in **Table 5.1**. Not all possible hydrolysis products are always detected, and γ-chaconine and β_1-solanine are in general rarely observed. More studies suggest that β_2-chaconine was directly transformed into solanidine, because no γ-chaconine was detected (Guseva and Paseshnichenko, 1957; Swain et al., 1978). The same hydrolysis pattern, where no γ-form was detected, was observed by Guseva and Paseshnichenko (1959), who tested the ability of potato sprouts to hydrolyze the two similar glycoalkaloids; solasonine and solamargine.

Table 5.1 Glycoalkaloid degradation products produced by enzyme extracts from various potato tissues.

Origin	**α-Chaconine**				**α-Solanine**				**Reference**
	β_1	β_2	γ	Solanidine	β_1	β_2	γ	Solanidine	
Sprouts	÷	✓	÷	✓	÷	✓	✓	✓	(Swain et al., 1978)
Sprouts	One β form		÷	✓	÷	✓	✓	✓	(Guseva and Paseshnichenko, 1957)
Leaves	No report of which products				No report of which products				(Guseva and Paseshnichenko, 1957)
Potato peel	✓	✓	No further degradation studied		✓[a]	✓	No further degradation studied		(Bushway et al., 1988; Bushway et al., 1990)
Tubers	✓	✓	✓	✓	÷	✓	÷	✓	(Swain et al., 1978)
Berries and blossom mixture	÷	✓	÷	✓	No degradation				(Filadelfi and Zitnak, 1982)

[a] Small amount, primary β-form was β_2.

The reported hydrolysis products differ between the tissue extracts. In one study, the enzyme mixture prepared from sprouts produced only β_2-chaconine and solanidine, while the extracts based on dormant tubers produced all four possible hydrolysis products (Swain et al., 1978). Opposite, it was observed that the sprout assay produced β_2-solanine, γ-solanine, and solanidine from α-solanine, while fewer hydrolysis products were produced from the tuber extract. Not all tissues hydrolyzed both potato glycoalkaloids equally; the blend of berries and blossom did only hydrolyze

α-chaconine (Filadelfi and Zitnak, 1982) and also the potato sprouts hydrolyzed α-chaconine more vigorously than α-solanine (Guseva and Paseshnichenko, 1957).

All of the above mentioned results are based on enzyme mixtures prepared from potato tissues, while reports of any of the hydrolysis products in intact plant tissues are few. β-Chaconine appears to be the only hydrolysis product detected in intact tissues, and this compound has been found in roots, sprouts, leaves/stem, and in tubers from varieties known to have a high glycoalkaloid content (Morris and Petermann, 1985; Friedman and Dao, 1992; Friedman and McDonald, 1995b; **Manuscript III**). In the sprouts, Friedman and McDonald (1995b, 1997) observed that all α-chaconine were converted to β_2-chaconine after a prolonged storage of sprouted potatoes at 3-4 ºC, while α-solanine was unaffected. In accordance, it was observed in **Manuscript III**, that β-chaconine was produced in decaying plant material, and that the α-chaconine concentration was faster declining than α-solanine. In the tomato plant, the degradation has primarily been studied in the fruits used for consumption, because they contain high amount of glycoalkaloids, when they are green and immature. In the fruits, almost all tomatine are enzymatic degraded to 3β-hydroxy-5α-pregn-16-en-20-one during ripening (Heftmann and Schwimmer, 1972; Friedman, 2002).

5.1.2 Microbial Degradation and Metabolite Formation

Only few studies have investigated the microbial degradation of the potato glycoalkaloids. Most of the studies cover potato pathogenic fungi, which degrade the glycoalkaloids as a detoxification mechanism (Morrissey and Osbourn, 1999). In general, the glycoalkaloids are degraded by removal of one or more of the carbohydrates, whereby the toxicity is reduced. *Fusarium caeruleum*, a cause of dry rot in potato, was reported to hydrolyze α-solanine into solanidine with β- and γ-solanine as intermediates (McKee, 1959). The blight fungus, *Phytophthora infestans*, performed a similar conversion (Holland and Taylor, 1979), but no intermediates were observed. Weltring et al. (1997) studied the degradation of both glycoalkaloids performed by two strains of another cause of potato dry rot, *Gibberella pulicaris*. α-Chaconine was by one strain converted to β_2-chaconine and further to solanidine, which was metabolized further to at least two other unknown products. This strain did also convert α-solanine, which was hydrolyzed into β_2- and γ-solanine. The other strain did only convert α-chaconine, and the first intermediate, β_2-chaconine, was only partly converted into solanidine. In another study, three strains of filamentous fungi did similarly degrade α-chaconine only (Oda et al., 2002). Further study of one of the strains, showed a conversion into β_1-chaconine, after which no further conversion proceeded. These strains were isolated from mould on surface of potato sprouts, but in contrast to the fungi used in the other studies, these three fungi strains (*Cladosporium cladosporioides*, *Penicillium* sp., *Plectosphaerella cucumerina*) were not reported to be pathogenic to potato plants.

All the above mentioned fungi, which to different extent possessed the ability to convert the glycoalkaloids into less toxic metabolites, were either known potato pathogens or isolated from potato tissue, and had as such shown their ability to live in close association with the potato plants. In **Manuscript IV,** the degradation of potato glycoalkaloids in groundwater sampled from below a potato field was investigated. The groundwater microorganisms did degrade both glycoalkaloids,

and degradation proceeded by stepwise removal of the carbohydrate units until the formation of solanidine. Solanidine was degraded too, but it was not possible to identify any further metabolites in the initial studies, where the solutions were analyzed by LC-TOF-MS. In contrast to the previous fungal studies, where β_2-solanine was formed from α-solanine, in this work β_1-solanine was produced. Bacterial growth was observed during the degradation, showing the glycoalkaloids to be partly converted into bacterial biomass.

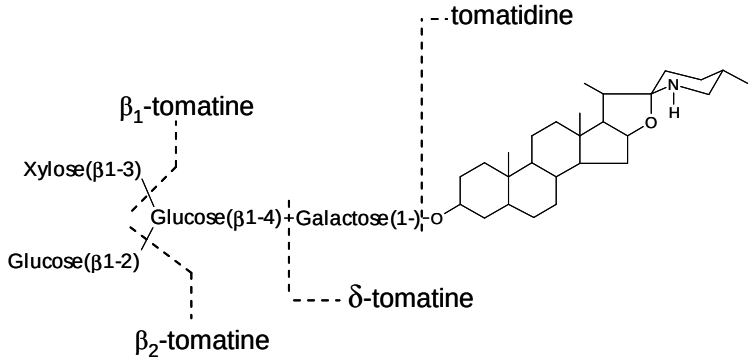

Figure 5.2 Degradation products from fungal degradation of α-tomatine

Other glycoalkaloids may also be detoxified by removal of one or more of the carbohydrate units. For α-tomatine, the reported end products are β_1-tomatine, β_2-tomatine, and tomatidine (Sandrock and VanEtten, 1998; Quidde et al., 1998; Friedman, 2002) (**Figure 5.2**), whereas reports of produced intermediates are sparse. The degradation to tomatidine appears as either sequential removal of the carbohydrate units or as a direct cleavage of the tetrasaccharide (Ford et al., 1977; Sandrock and VanEtten, 1998). A comprehensive study was performed by Sandrock and VanEtten (1998), who examined the ability of 23 fungal strains to degrade α-tomatine. Both tomato pathogens, pathogens of other non-tomato hosts, and two saprophytic fungi were included in this study. Almost all tomato pathogens did convert α-tomatine into β_2-tomatine or tomatidine. In contrast, only one non-tomato-pathogen was able to convert α-tomatine. Further investigation of the degradation process for one of the fungi, revealed the formation of another metabolite in-between the occurrence of β_2-tomatine and tomatidine; this metabolite was identified as δ-tomatine (**Figure 5.2**) (Sandrock and VanEtten, 1998). No pathogens have been reported to further degrade the aglycone, but this ability has been documented for some other fungi (Belič and Sočič, 1972; Gaberc-Porekar et al., 1983).

5.1.3 Degradation in Soil

Degradation in soil is often studied by dissipation studies, where soil is spiked with the compound of interest. At certain times, the remaining amount is determined, and the amount of no longer extractable compound is assigned to be degraded. Degradation of organic compounds is often described by simple first-order kinetics (Eq. 5.1, **Table 5.2**). In other cases, the degradation follows a bi-phasic pattern, where a fast initial dissipation is followed by a decrease in the dissipation rate,

Table 5.2 Kinetic expressions used for describing the dissipation in soil.

	Kinetic expression	DT_{50}	Eq.
1.order kinetics	$C_t = C_0 \times \exp(-k \times t)$	ln2 / k	5.1
2 × 1.order kinetics	$C_t = a \times \exp(-k_1 \times t) + (1-a) \times \exp(-k_2 \times t)$	Iterative procedure	5.2

C_0 and C_t are the concentrations of the compound present in the soil at time 0 and t, k values are rate constants (/day), t is time (days), and the constant *a* expresses the concentration of the compound degraded by the fast process.

Table 5.3 Degradation kinetics and obtained half-lives (DT_{50}) of selected natural compounds.

Compound	DT_{50}	Temp.	Water content	Kinetics	Reference
DIMBOA	31 min	25 ºC	2:1 (soil:water)	1.order	(Macías et al., 2004)
DIBOA-glucose	1 day	25 ºC	2:1 (soil:water)	1.order	(Macías et al., 2005)
DIBOA	1-2.6 day	25 ºC	2:1 (soil:water)	1.order	(Macías et al., 2005)
Glucosinolates[a]	3.5-15.5 h	20 ºC	1:1 (soil:water)	Logistic	(Gimsing et al., 2006)
Dhurrin	0.25-2h (topsoil) 0.2-12 day (subsoil)	22 ºC	1:1 (soil:water)	1.order	(Johansen et al., 2007)
Linamarin / lotaustralin	1.5-35 h	2.5 ºC	Leaching experiment	1.order	(Bjarnholt et al., 2008)
Ptaquiloside	0.3-7.5 day	25 ºC	1:1 (soil:water)	1.order or 2 × 1.order	(Rasmussen et al., 2005)
Artemisinin	0.9-4.2 day	21 ºC	60% of field capacity	2 × 1.order	(Jessing et al., 2008)
Ochratoxin A	0.2-1.2 day	18 ºC	60% of field capacity	2 × 1.order	(Mortensen et al., 2006)
Aflatoxin B1	4.1 day	25 ºC		1.order	(Accinelli et al., 2008)
Zearalenone	6.4-16 day	18 ºC	60% of field capacity	2 × 1.order	(Mortensen et al., 2006)
Potato glycoalkaloids	1.8-4.1 day 4.7-8.7 day	15 ºC 5 ºC	60% of field capacity	2 × 1.order	**Manuscript IV**
Strychnine	24-27 day	25 ºC	75% of field capacity	1. order[b]	(Starr et al., 1996)

[a] but-3-enyl, 2-hydroxy-but-3-enyl, benzyl, and phenethyl

[b] after initial lag-phase

and this pattern can be described by a sum of two first-order expressions (Eq. 5.2, **Table 5.2**). Both expressions are frequently used for pesticide degradation (Beulke and Brown, 2001), and the expressions have also found use for natural compounds; Macías et al. (2004; 2005), for example, used eq. 5.1 for description of dissipation of the benzoxazinoids, DIBOA-Glc, DIBOA, and DIMBOA. Eq. 5.2 has been used for description of dissipation of ochratoxin A, zearalenone, ptaquiloside, and artemisinin in soil (Mortensen et al., 2006; Ovesen et al., 2008; Jessing et al., 2008). The half-lives (DT_{50}) can be derived from the expressions (**Table 5.2**), and they are used in

risk assessment. Obtained half-lives for a range of natural compounds are presented in **Table 5.3**. The obtained DT_{50} values range from a few hours for e.g. dhurrin and the glucosinolates up to 24-27 days for strychnine. The latter half-life did, however, include an initial three week lag-phase. The values are relatively low compared to the upper limit of 90 days for a compound to be approved by the Danish Environmental Protection Agency as a pesticide (Miljøstyrelsen, 1999).

Degradation in Soil - Glycoalkaloids

For the glycoalkaloids, only two dissipation studies have been performed. In a previous work by Jensen et al. (2007), slow dissipation was observed in a sandy soil at 5 ºC, where most glycoalkaloids remained in the soil after 36 days of incubation. When a potato extract in stead of the pure compound was used for spiking, a faster dissipation, where most of the α-solanine dissipated within 26 days, was observed. The results indicated the faster dissipation to be due to an enhanced degradation; either due to the enzymes in the potato extract or due to an increased microbial activity because of the easily available nutrients provided with the potato extract. No half-lives were derived from this work. In the present work (**Manuscript IV**), degradation of pure α-solanine was followed in three soils at three temperatures, at field moisture, and described using eq. 5.2. The results showed that the degradation was fastest in the sandy soil, while slower degradation was observed in the soils with more organic matter or clay. Half-lives in the range from 2-4 days were obtained for the three soils at 15 ºC, while somewhat higher half-lives of 5-9 days were found at 5 ºC in the sandy top- and subsoil. The variation in the observed degradation rates between the two studies may possibly be due to differences in soil properties or microbial activity. In spite of the relatively short half-lives, residuals were still detected in the present work at the end of the experiment after 42 days, as a result of a low second degradation rate.

A glucose moiety is a part of the structure in many of the natural compounds (dhurrin, linamarin, lotaustralin, ptaquiloside, glucosinolates, DIMBOA-Glc). The glucose is here linked either by a glycosidic linkage or as a thioglucose. In all cases, the glucose moiety is detached during the first degradation step. The half-lives for these types of compounds are in general relatively short, ranging from hours to a few days (**Table 5.3**), although higher DT_{50} values for ptaquiloside and dhurrin were obtained in a few subsoils. Comparing the half-lives for the glycoalkaloids to the other carbohydrate containing compounds, the glycoalkaloids are among the slower degraded. The obtained half-lives are though still within the range previously observed for the other compounds, when the temperature used in the studies is taken into consideration. Compared to obtained half-lives for a well-known pesticide, MCPA, (4-16 days, 20 ºC) (Thorstensen and Lode, 2001), which is thought as easily degradable, the obtained half-lives of the glycoalkaloids are still relatively short.

5.2 Sorption

Sorption reduces the mobility of a compound in soil, thereby limiting the risk of leaching to the groundwater. Organic matter and clay particles are dominating sorbents in the soil. Cationic organic compounds can sorb to negatively-charged sites on the clay particles or in the soil organic mater, and this type of sorption is related to the cation exchange capacity of the soil. Non-ionic organic

compounds sorb primarily to the less polar parts of the soil organic matter. The sorption process is in principle reversible, whereby the equilibrium is kept at all times.

The Freundlich isotherm is commonly used to describe sorption:

$$C_{soil} = K_f \times C_{water}^{n} \qquad \text{[Eq. 5.3]}$$

where C_{soil} and C_{water} are the concentrations of the compound in the soil and in the soil water, respectively, K_f is the Freundlich distribution coefficient, and *n* is a constant. If *n* equals one, the equation is reduced to a linear sorption isotherm, which is also commonly used;

$$C_{soil} = K_d \times C_{water} \qquad \text{[Eq. 5.4]}$$

where K_d is the linear distribution coefficient. K_d is soil specific and depends on the sorption properties of the soil. It can be converted into a more general parameter, K_{oc}, which is a distribution coefficient between the soil water and the organic carbon in the soil:

$$K_d = K_{oc} \times f_{oc} \qquad \text{[Eq. 5.5]}$$

where f_{oc} is the fraction of organic carbon in the soil.

5.2.1 Sorption - Glycoalkaloids

No sorption studies of the potato glycoalkaloids have been published. Both glycoalkaloids contain a tertiary amine, which is protonated at acidic pH. α-Solanine has a pK_a value of 6.7, while no pK_a value has been reported for α-chaconine; a value of same size can be expected due to the structural similarities of the compounds. The glycoalkaloids will therefore be present in both cationic and uncharged form in most agricultural soils. Sorption of another alkaloid, strychnine, which is also present in both forms in soil, has been investigated (Starr et al., 1996). A correlation between the cation exchange capacity and the sorption was observed. Furthermore, indications of a greater degree of partitioning into the soil organic matter were seen at more alkaline pH, when a higher proportion of the compound was uncharged. Hence, it was suggested that adsorption of the protonated form through ion-exchange processes as well as partitioning of the uncharged form into the soil organic matter were both involved in the sorption of strychnine. This may possible be the case for the glycoalkaloids too. However, beside the amine group, the structures of strychnine and the glycoalkaloids are not very alike, why any comparison should be taken with caution.

The partitioning into organic matter can be estimated by eq. 5.5, and estimated values for K_{oc} for the two glycoalkaloids were given in **Table 2.1**. For at typical agricultural soil (f_{oc} = 2%), this will lead to K_d values of 250-400 L/kg for the two glycoalkaloids. Subsoils will typically have lower organic matter content, e.g. 0.1%, and consequently K_d will be lower (13-20 L/kg). Hence, the major proportion of the glycoalkaloids will be found sorbed to the soil. However, this only takes sorption of the uncharged fraction of the glycoalkaloids into the organic matter into account. If a considerable proportion of the glycoalkaloid is found as a cation, as it would be in slightly acidic soils, this may lead to reduced sorption into the organic matter. In contrast, the cationic form may be adsorbed through ion exchange processes, which may be of importance in particular in soils with

high cation exchange capacities. This type of sorption was studied for the protonated form of another glycoalkaloid, α-tomatine, which was shown to sorb strongly to different types of clay (Johns, 1986). Overall, it is difficult to predict, from the structure alone, how much the sorption into the soil organic matter is reduced for the cation, and how strongly the adsorption through ion-exchange processes is. It would thus be preferable to perform studies in the laboratory to investigate the size and mode of sorption for the glycoalkaloids and obtain empiric results. Further, sorption of the degradation products, the β- and γ-compounds, into the soil organic matter is expected to be stronger, because of their smaller carbohydrate moieties. The aglycone, solanidine, without any carbohydrate units at all, is expected to have the strongest sorption of all, as reflected in the estimated log K_{oc} of 6.1 (**Table 2.1**).

A range of partition coefficients for other natural compounds are presented in **Table 5.4**. Some compounds, as strychnine and zearalenone, have distribution coefficients in the same magnitude as the glycoalkaloids, while others, as ptaquiloside, the cyanogenic glycosides (dhurrin, linamarin, lotaustralin), and the glucosinolates, are almost solely found in the soil water.

Table 5.4 Experimental and estimated distribution coefficients for selected natural compounds.

Compound	**Distribution coefficients**			**Soils**	**Reference**
	K_{oc} (L/kg)	**K_d (L/kg)**	**K_f**		
Dhurrin		No sorption (exp)		Five soils; top- and subsoils	(Johansen et al., 2007)
Linamarin/ Lotaustralin		No sorption (exp)		Two soils (sandy and loamy)	(Bjarnholt et al., 2008)
Glucosinolates[a]		No sorption (exp)		Two soils; top- and subsoils	(Gimsing et al., 2006)
Ptaquiloside			0.01-0.22[b] (exp)	10 soils, top- and subsoils	(Rasmussen et al., 2005)
Artemisinin	$10^{2.51}$ (est)				(Jessing et al., 2008)
Juglone	$10^{2.56}$- $10^{3.44}$ (exp)	3.0-3.7 (exp)		Top- and subsoil (silty loam)	(von Kiparski et al., 2007)
Ochratoxin A	$10^{3.29}$ (est)				(Mortensen et al., 2006)
Zearalenone	$10^{3.89}$ (est)				(Mortensen et al., 2006)
Potato glycoalkaloids	$10^{4.1}$- $10^{4.3}$ (est)	13-400 (est)		Top- and subsoil	This project
Strychnine	$10^{3.97}$- $10^{4.20}$ (exp)		40-169[c] (exp) 55-173[d] (exp)	Four soils, topsoils Four soils	(Starr et al., 1996) (Kookana et al., 1997)

[a] but-3-enyl, 2-hydroxy-but-3-enyl, benzyl, phenethyl
[b] n = 0.65-1.00, [c] n = 0.75-0.93, [d] n = 0.52-0.66

5.3 Field Studies

Investigations in the field are highly important in order to determine the fate of a compound. In the field, processes as sorption, degradation and leaching will all be included, and the system is exposed to naturally varying conditions regarding e.g. temperature, precipitation, and daylight. In addition, the application pattern of a natural compound will be realistic. The application pattern is different from that of a pesticide, which is applied by the farmer to the field in a known amount at a certain time. The natural compounds may be released by different mechanisms from the plants; volatilization, leaching from plant parts, decomposition of plant residues, or root exudation (Chou, 1999) and the amount released from the plant is uncertain. The plants are also present in the field for a long period of time. This may overall lead to a more continuous application process.

Table 5.5 Content of selected natural compounds in soil and water. All data for soil are based on dry weight.

Compound	Soil	Water	Reference
Thujone	169-1,200 mg/kg (O)[a] 0.0012-0.0039 mg/kg		(Strobel et al., 2005)
Juglone	0.0-5.7 mg/kg		(Ponder and Tadros, 1985; De Scisciolo et al., 1990; Jose and Gillespie, 1998; von Kiparski et al., 2007)
Artemisinin	0-2 cm: 0.16-11.7 mg/kg 2-5 cm: 0.0-0.5 mg/kg		(Jessing et al., 2008)
Aflatoxin B1	0.0006-0.0055 mg/kg		(Accinelli, et al., 2008)
Potato glycoalkaloids	0.0-2.8 mg/kg	No findings	**Manuscript III**
Ptaquiloside	0.1-8.5 mg/kg (O)[a] 0.007-5.0 mg/kg	Soil water (90 cm): 0-7 µg/L Surface wells: 30-45 µg/L	(Rasmussen et al., 2003a; Rasmussen et al., 2003b; Rasmussen et al., 2005; Engel et al., 2007)
Zearalenone	0.000-0.0075 mg/kg	River water: below LOQ[b]	(Hartmann et al., 2008; Bucheli et al., 2008)
Deoxynivalenol		River water: up to 22 ng/L	(Bucheli et al., 2008)
Batatasin-III		Soil solution: App. 50-200 µg/L Snowmelt: 21-54 µg/L	(Wallstedt et al., 2000)

[a] O-horizon
[b] Limit of quantification

Table 5.5 shows a range of natural compounds which have been detected in soil and water. The observed soil concentrations should be divided between concentrations found in the O-horizon and in the subsequent mineral soil layers. This is due to the fact that plant material possible including the natural compounds may be left as debris on the top of the soil and turn into O-horizon. Thus, higher concentrations can be expected in the organic layer, as reported for e.g. thujone and

ptaquiloside, where concentrations up to 1200 mg/kg and 8.5 mg/kg, respectively, were found in the organic soil layers (Rasmussen et al., 2003a; Strobel et al., 2005). The thujone concentration in the upper mineral soil was approximately a factor of 160 lower than the average concentration in the O-horizon. The general concentrations reported for the mineral soil are ranging from a few µg/kg (zearalenone, aflatoxin B1, and thujone) to 5-12 mg/kg (ptaquiloside, juglone, and artemisinin). Furthermore, a decrease in concentration with depth has been observed for both ptaquiloside, juglone, and artemisinin (Ponder and Tadros, 1985; Engel et al., 2007; Jessing et al., 2008). The range of concentrations reported for a single compound may vary widely; this can be explained by spatial or seasonal variation, the amount originally applied, or differences between the study sites. For juglone, e.g. a spatial variation was related to the distance from the walnut tree releasing the compound (Jose and Gillespie, 1998). Seasonal variation has been investigated for batatasin-III and juglone (De Scisciolo et al., 1990; Jose and Gillespie, 1998; Wallstedt et al., 2000), but as only three sampling times were used, trends are difficult to derive. The variation between different study sites are illustrated by Rasmussen et al. (2003b), who measured ptaquiloside in soil from 20 different Danish sites; the concentrations differed up to a factor of 100. Also the amount applied to the soil may vary; e.g. the ptaquiloside concentration and amount in the plants were found to vary over a factor of 20-25. Beside the above mentioned reasons, variation in the reported concentrations may also be influenced by different sampling strategies (e.g. sampling depth) or extraction procedures.

Only a few of the natural compounds have been found in various water samples (**Table 5.5**), however for many of the compounds there exists no reports of analysis of any water samples at all. The highly water soluble compound ptaquiloside have been detected in soil water from 90 cm and in surface wells (Rasmussen et al., 2005). Also, the two mycotoxins, deoxynivalenol and zearalenone, have been detected in low concentrations (ng/L) in river water (Bucheli et al., 2008).

5.3.1 Field study - Glycoalkaloids

For the potato glycoalkaloids, the present work is the first field study ever done. The glycoalkaloid content in plants, soil, and groundwater from a potato field was followed during the growth season and the following winter. Both seasonal and spatial variation was investigated by sampling of soil at eight dates and by analysis of ten individual soil samples for each sampling date. The glycoalkaloids were detected in the upper soil layer (2-3 cm) and a seasonal variation was observed (**Figure 5.3**). The highest concentrations of up to 2.8 mg/kg were detected during autumn. This is within the high end of the range found for other natural compounds. The glycoalkaloids were still detected in the following March in spite of no further application of glycoalkaloids during the winter. Additionally, a very high spatial variation was observed, e.g. concentrations in November ranged about a factor of 60, this illustrates the importance of a well thought sampling strategy. No glycoalkaloids were found by analysis of the groundwater from 2-4 m depth sampled from May through November. The results of the field study are further presented and discussed in **Manuscript III**.

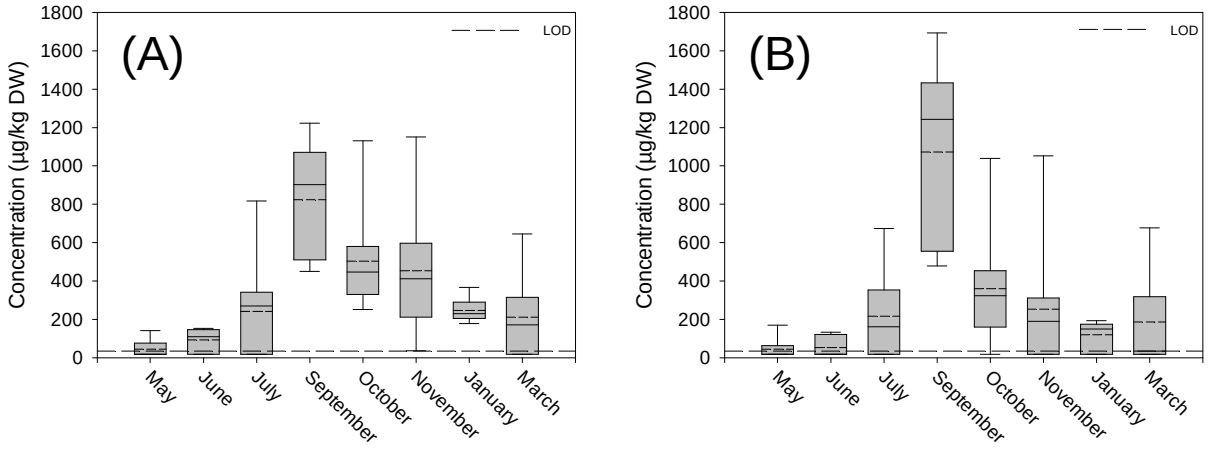

Figure 5.3 Concentrations of the glycoalkaloids in the soil during the season. Detection limits (LOD) are shown as long dashed lines. The boundaries of the box indicate the 25th and the 75th percentile, respectively. Error bars indicate the 90th and 10th percentiles. In the box, the mean is presented by a solid line and the median by short dash line. (A) α-Solanine. (B) α-Chaconine. DW = dry weight. From **Manuscript III**.

5.4 Biological Effect Study

A small initial study was performed to investigate, if the presence of glycoalkaloids in the soil affected the mineralization of other compounds. This study is not included in any of the manuscripts, why it is presented in more details here. Mineralization was followed for three selected compounds (acetate, MCPA, and mancozeb). Mancozeb is a fungicide used in potato cropping, MCPA is a commonly used pesticide, and acetate was chosen to represent the general microbial activity. Three soils from the field location at Fladerne Bæk were used; a potato field, a spring barley field, which was used for potato cropping the previous year, and a soil planted by spruce. Soils were spiked with a ^{14}C-labelled compound (acetate, MCPA, or mancozeb) and a glycoalkaloid mixture (1:1 of α-chaconine and α-solanine) in concentrations of about 0, 1, 12, and 60 mg/kg DW soil. The concentrations were selected to cover a real situation, with concentration levels similar to what was detected in the soil (up to 2.8 mg/kg DW) and a worst case scenario where all glycoalkaloids present in the plants were momentarily released to the upper 2 cm of soil (around 100 mg/kg DW). Mineralization was followed for 13 days by collection of the produced $^{14}CO_2$ in 0.5 M NaOH. The solution of NaOH was changed regularly, and the amount of ^{14}C was hereafter determined using a liquid scintillation counter. The results showed that the mineralization of acetate and mancozeb did not differ between the various glycoalkaloid concentrations or between the three soils. For MCPA, no difference was observed between the different added glycoalkaloid concentrations either, whereas a difference was observed between the three soils, where a slower mineralization was found for the field used for potato growing (**Figure 5.4**). This was not investigated further during this project, but it could be of interest to investigate if the lower mineralization was related to some aspects of the potato growing. Overall, no effect of the

glycoalkaloids was found in this little study – neither for glycoalkaloids in natural concentrations nor in the concentration representing a worst case scenario.

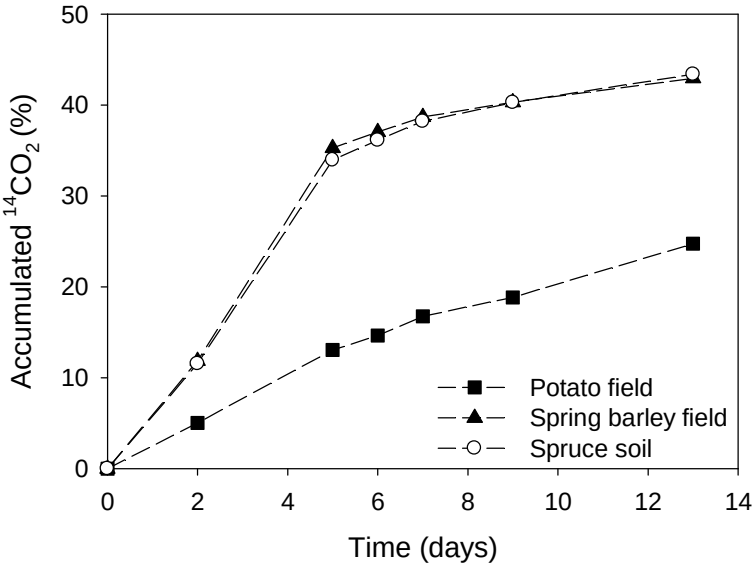

Figure 5.4 Mineralization of MCPA in three soils with a glycoalkaloid concentration of up to 60 mg/kg DW soil.

6 Conclusions and Perspectives

6.1 Analytical Study

A quantitative HPLC-TOF-MS method has been developed for determination of the two glycoalkaloids; α-chaconine and α-solanine, and their common aglycone, solanidine. The method was developed as a supplement to the ordinary HPLC-UV method in order to enable work with low concentration samples and in order to include degradation products in the analysis. The optimized method showed good performance in respect to repeatability and linear ranges, and the obtained detection limits were in the range 2.2-4.7 µg/L. Overall, the work showed the HPLC-TOF-MS to be a powerful tool for quantitative studies of glycoalkaloids including unknown metabolites. Further, a single quadrupole-MS was included in some of the studies, where the obtained detection limits were lowered to 0.1-0.5 µg/L. In conclusion, the change from UV to a TOF-MS or a single quadrupole-MS for detection lowered the detection limits about a factor of 100 and 1,000, respectively, and permitted inclusion of metabolites in the analysis.

6.2 Fate Study

The potato glycoalkaloids were found to be degraded by soil and groundwater microorganisms. Metabolite formation was investigated in groundwater, and the first metabolites formed were the less toxic β- and γ-compounds and solanidine. Solanidine was further degraded into unknown metabolites. The found metabolites are expected to be of less risk than the main glycoalkaloids because of their lower toxicity and higher sorption. In various soils, including one subsoil, investigated at relevant soil temperatures (5-20 ºC), half-lives for the two glycoalkaloids were found to be in the order of 2-9 days. This is within the high range of half-lives found for other natural toxins, but overall, relatively short compared to e.g. many pesticides. Residuals were though observed to remain in the soil for a longer period of time (i.e. month(s)) in both incubation and field studies.

In the field study, a high potential load of glycoalkaloids was found in the plants (25 kg/ha). In spite of this, only a minor fraction of the maximum plant content (2%) was found in the upper soil layer. It was shown that the glycoalkaloids were degraded in the plants during senescence, why it is suggested that the major dissipation route for the glycoalkaloids in the plants were degradation within the plants rather than a transfer to the soil. The highest glycoalkaloid concentration in the soil was detected during autumn, while the highest plant content was detected during summer. Tubers left in the field after harvest contained glycoalkaloids as well, but this amount could not account for the content found in the soil. From these observations, the major transfer route from the plants to the soil appeared to be transfer from the plants during plant senescence. The field work was performed on a sandy soil with heavy irrigation and high percolation, and the general risk of leaching from this soil was considered to be high. However, in spite of the vulnerability of the soil and the high plant content of the glycoalkaloids, the analysis of the upper groundwater during the growth season showed no trace of glycoalkaloids. Hence, the results showed the risk of

glycoalkaloid leaching to the groundwater to be low. Furthermore, soil concentrations of glycoalkaloids, ranging from a real scenario to a worst case scenario, showed no effect on the microbial activity or the ability to mineralize selected pesticides. Hence, there was no indication of effect on the soil microorganisms; however it should be emphasized that this was a small initial study, why further studies would be needed before any conclusion upon the effects of the glycoalkaloids in the soil can be drawn. Also, effects on other soil organisms should be tested, as the literature show that glycoalkaloids can affect organisms as insects and snails.

In conclusion, a relatively small proportion of the potato glycoalkaloids present in the plants are transferred to the soil. The soil and groundwater microorganisms are able to degrade the glycoalkaloids, and the initial degradation is relatively fast. However, glycoalkaloid residuals can be detected in the very topsoil for a long period, but despite of that there is no indication of leaching to the groundwater. From the results obtained in this work, the overall conclusion is that the risk of glycoalkaloid leaching is low.

6.3 Perspectives

The present work showed that the glycoalkaloids were degradable by the microorganisms in the terrestrial environment, and that only a minor proportion of the glycoalkaloids produced in the plants were found in the soil. Additionally, no glycoalkaloids were detected in the groundwater in spite of the vulnerability of the scenario present for potato growing; sandy soil, high irrigation, and high percolation. From the overall results, the risk of the glycoalkaloids in the terrestrial environment in terms of leaching appears to be low. Some questions, however, still remain in order to fully clarify if the glycoalkaloids may possess a risk in the terrestrial environment. Further, additional questions may be asked in order to obtain a better understanding of the processes.

One point, which needs further attention, is the sorption of the glycoalkaloids in the soil. High sorption is expected from the properties of the glycoalkaloids, as discussed previously. The presence of both cationic and uncharged forms of the glycoalkaloids in most soils will possibly influence the sorption, and this influence needs to be investigated; i.e. is the cationic form sorbed as strongly as the uncharged form? To answer this question, sorption studies at various pH could be performed, and the sorption of the glycoalkaloids to the individual sorbents, organic matter and clay, could be investigated.

α-Solanine and α-chaconine were both found to be degraded to the aglycone, solanidine, in the present work. Further degradation of solanidine was observed in the groundwater; however, the degradation pathway from solanidine still remains to be elucidated. The literature report that solanidine has low estrogenic effects, why further investigation of the complete degradation of the glycoalkaloids is of interest.

The effect of the glycoalkaloids in the soil is another subject, which has been only touched lightly in the present work. An initial study was performed, but further work is needed in order to investigate a possible effect of glycoalkaloids on microorganisms. Furthermore, literature show that the

glycoalkaloids affect organisms as insects and snails, why the found soil concentrations should be tested upon other soil organisms.

The dissipation routes of the glycoalkaloids in the plants are another aspect, which would be of interest to study in more detail. Further studies of the degradation within the plant during senescence could be performed in order to determine the degradation pathway. Additionally, studies of a possible degradation within the left and partly decomposed tubers in the field could be performed. The transfer process from the plant to the soil has been partly resolved in the present work. The field study indicated that the glycoalkaloids were mainly transferred to the soil during plant senescence. However, several processes are integrated in a field study, and studies under controlled conditions in the lab would give further insight in the transfer process. Thus, it would be of interest to study the release of the compounds under controlled conditions in order to quantify the amount released during the different processes (e.g. rain-off from the leaves, release during plant senescence, or release from tubers). Hereby, further knowledge of when a possible risk in the terrestrial environment is present would be gained.

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